

# **The role of Ack1 in TRAIL receptor signalling**

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## **Declaration of Authenticity**

I, Emma Linderöth confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.



## **Contributions**

The following experiments were contributions made by others:

- I. The immunocytochemistry experiment in Figure 21A was performed by Giulia Pilia.
- II. The immunocytochemistry experiment in Figure 25 was performed by Giulia Pilia.
- III. All confocal images were acquired by the assistance of Andres Vicente at the London Research Institute, Cancer Research UK.

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## **Abstract**

The Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is one of the most recently identified death inducing ligands of the TNF cytokine family. TRAIL induces apoptosis in most cancer cells, whereas the majority of normal cells are resistant. TRAIL receptor agonists are therefore considered to be a promising anti-cancer therapeutic. However, because many cancer cells develop resistance to TRAIL, understanding the mechanisms by which resistance is acquired will be critical for the therapeutic use of TRAIL in cancer therapy.

We have discovered that Activated Cdc42-associated kinase 1 (Ack1) is required for TRAIL induced apoptosis in human epithelial cells. Ack1 is a non-receptor tyrosine kinase with numerous protein-protein interaction domains, suggested to have a role in several cellular processes such as trafficking, endocytosis and cell motility.

Knockdown of Ack1 in various epithelial cell lines leads to significantly impaired TRAIL induced apoptosis as evident by reduced cleavage of Caspase-8 and -3 and surface exposure of phosphatidylserine. Exploring the underlying mechanism we found that Ack1 knockdown leads to impaired TRAIL induced clustering of TRAIL-R1 and a reduction in the recruitment of Caspase-8 to the DISC complex, essential for death inducing signal transduction.

Translocation of the TRAIL receptors to lipid rafts in the plasma membrane have been suggested to be crucial for TRAIL receptor dynamics and downstream signalling following TRAIL ligand binding. In this work we show that Ack1 is required for the translocation of the TRAIL receptors to the lipid rafts.

In this thesis, a novel regulatory role of Ack1 in apoptosis, death receptor signalling and lipid raft trafficking is presented, contributing further to the understanding of the molecular regulation of TRAIL receptor signalling.

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## Abbreviations

Ack1	Activated Cdc42-associated kinase 1
AIM-100	4-amino-5,6-biaryl-furo[2,3-d]pyrimidine
AJ	Adherens junction
AP2	Adaptor protein 2
APS	Ammonium persulfate
Apaf-1	Apoptotic protease activating factor 1
APP	$\beta$ -amyloid precursor protein
AR	Androgen receptor
Ark-1	A Ras-regulating Kinase 1
Biotin-7-NHS	D-biotinoyl- $\epsilon$ -aminocaproic acid-N-hydroxysuccinimide ester
BSA	Bovine serum albumin
Cav1	Caveolin-1
Cdc42	Cell division cycle 42, GTP binding protein
c-FLIP <sub>L/R/S</sub>	Cellular FLICE-like inhibitory protein long/ Raji/ short
CHC	Clathrin heavy chain
CPZ	Chlorpromazine
Co-IP	Co-immunoprecipitation
CRIB	Cdc42/Rac interactive binding domain
CSD	Caveolin scaffolding domain
CRD	Cysteine rich domain
Cyt <i>c</i>	Cytochrome <i>c</i>
DAPI	4',6-diamidino-2-phenylindole
DcR1	Decoy receptor 1
DcR2	Decoy receptor 2
DD	Death domain
DED	Death effector domain
DISC	Death inducing signalling complex
DR4	Death receptor 4
DR5	Death receptor 5
EBD	EGFR-binding domain

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transformation
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
FADD	Fas-Associated protein with Death Domain
FasL	Fas ligand
FBS	Fetal bovine serum
FUT3	Fucosyltransferase 3
GALNT14	<i>N</i> -acetyl galactosamine transferase 14
GAP	GTPase activating protein
GDI	Guanine nucleotide-dissociation inhibitor
GEF	Guanine nucleotide exchange factor
GJ	Gap junction
GPI	Glycophosphatidylinositol
h	Hour
HRP	Horseradish peroxidase
IAP	Inhibitors of apoptosis
ICH	Immunohistochemistry
IKK	I $\kappa$ B kinase
KD	Kinase dead
LB	Luria broth
LRR	Lysine rich region
Mig6	Mitogen inducible gene 6
Min	Minute
MAPK	Mitogen-activated protein kinase
MDCK	Madin Darby canine kidney
MFI	Mean-fluorescence intensity
MOMP	Mitochondrial outer membrane permeabilisation
Na <sup>+</sup> /K <sup>+</sup> ATPase	Sodium-potassium adenosine triphosphatase
Nedd4	Neural precursor cell expressed and developmentally down regulated 4
NF $\kappa$ B	Nuclear factor- $\kappa$ B

OPG	Osteoprotegerin
ORF	Open reading frame
PAK	p21-activated protein kinase
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PLAD	Pre-ligand-binding assembly domain
PMSF	Phenylmethylsulfonyl fluoride
PNK	Polynucleotide kinase
PS	Phosphatidylserine
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family, member A
Rpm	Revolutions per minute
RT	Room temperature
RTK	Receptor tyrosine kinase
SAM	Sterile $\alpha$ -motif
SDS	Sodium dodecyl sulphate
SH2	Src homology 2
SH3	Src homology 3
SMAC	Second mitochondria-derived activator of caspases
SNX9	Sortin nexin 9
Src	Sarcoma
tBid	Truncated Bid
TEMED	N, N, N', N'-tetramethylethylenediamine
TJ	Tight junction
TNF	Tumour necrosis factor
TNFR1	Tumour necrosis factor receptor 1
Tnk2	Tyrosine kinase, non-receptor 2
TRADD	Tumour necrosis factor receptor type 1-associated death domain protein
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R1	TRAIL receptor 1

TRAIL-R2	TRAIL receptor 2
UBA	Ubiquitin associated
WASP	Wiskott–Aldrich syndrome protein
WIBR	Wolfson Institute for Biomedical Research
Wwox	WW domain containing oxidoreductase
ZO-1	Zona occludens- 1

# Chapter 1 - Introduction

## 1.1 Overview of apoptosis

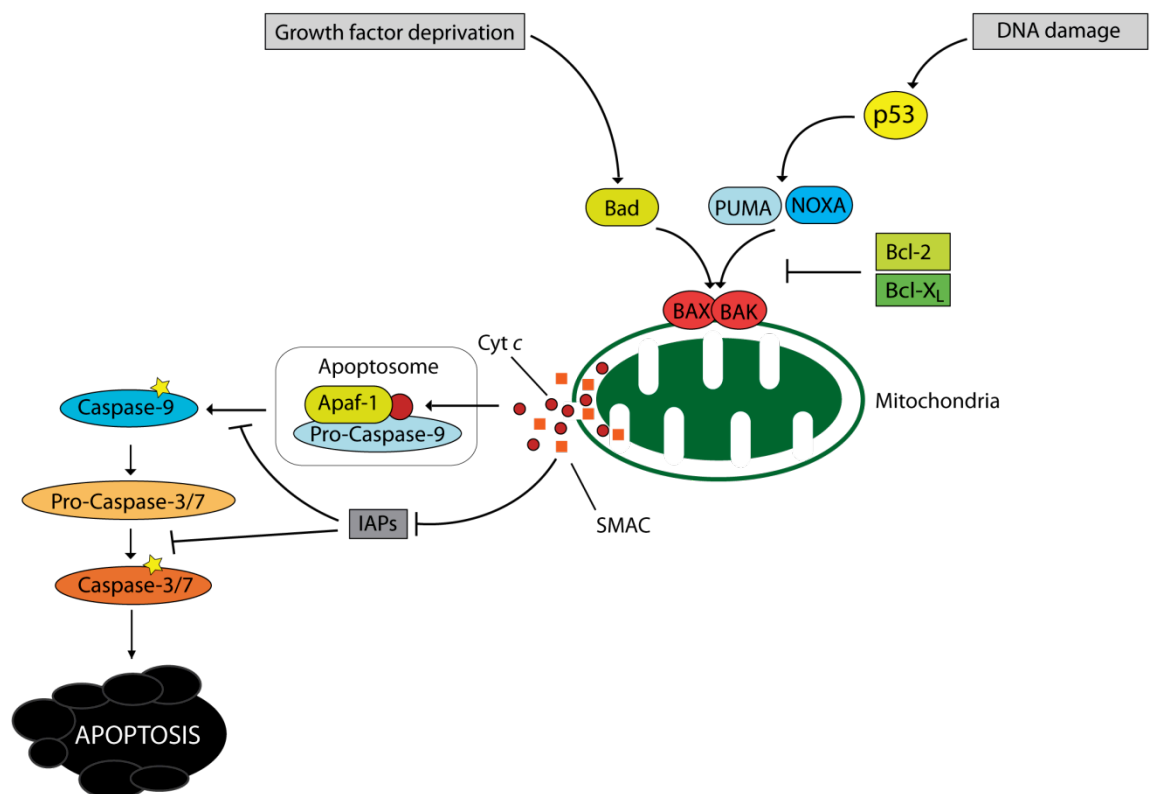
Apoptosis is an important process regulating cellular homeostasis, development and immune surveillance. Deficiency in apoptosis is one of the key contributing factors in cancer and also occurs in some autoimmune disorders. In contrast, excessive apoptosis leading to loss of essential cells takes place in neurodegenerative diseases, such as Alzheimer's [1]. Some of the hallmarks of apoptosis are cell shrinkage, chromatin condensation, DNA fragmentation [2], membrane blebbing, and exposure of the phospholipid, phosphatidylserine (PS) in the outer leaflet of the plasma membrane [3]. In contrast to necrosis, apoptosis does not induce an inflammatory response, and only individual cells are affected by apoptosis *in vivo*.

The main executors of apoptosis are a group of cysteine proteases called initiator and effector caspases [4, 5]. Caspases are constitutively expressed as inactive proenzymes (zymogens) and require proteolytic processing at specific aspartic acid residues in order to gain full activity. The caspases are activated in a hierarchical fashion; apoptotic signals first activate initiator caspases (caspase-2, -8, -9 and -10). Once activated the initiator caspases cleave and activate the effector caspases (caspase-3, -6 and -7), which in turn are able to cleave a wide range of vitally important cellular substrates in the cell, finally leading to cell death. Apoptotic signalling can be divided into two main pathways; the mitochondrial pathway and the death receptor pathway [6].

## 1.2 The mitochondrial pathway

The mitochondrial pathway, also known as the intrinsic pathway, is triggered by cellular stresses such as DNA-damage, starvation and hypoxia. The pathway is primarily regulated by the pro- and anti-apoptotic Bcl-2 family of proteins. The BH3-only subfamily of the Bcl-2 family, such as Bad, NOVA and PUMA, function as cellular sensors of damage or stress (p53 activation etc.) and can activate BAX and BAK, leading to mitochondrial outer membrane

permeabilisation (MOMP). In contrast, the anti-apoptotic Bcl-2 proteins, BCL-X<sub>L</sub> and Bcl-2 inhibit the activity of BAX and BAK thereby preventing MOMP. Permeabilisation of the mitochondria leads to the release of cytochrome c (cyt c) [7-9] and the protein second mitochondria-derived activator of caspases (SMAC) [10] into the cytosol. SMAC promotes apoptosis by binding and inactivating a family of endogenous inhibitors of apoptosis, called Inhibitors of Apoptosis (IAP). Released cyt c interacts with the adaptor protein, Apoptotic protease activating factor-1 (Apaf-1), and inactive pro-caspase-9 molecules, forming a complex called the apoptosome. Assembly of the apoptosome leads to cleavage and activation of caspase-9 which in turn activates effector caspases, such as caspase-3 and -7, subsequently leading to apoptosis.



**Figure 1. The mitochondrial pathway**

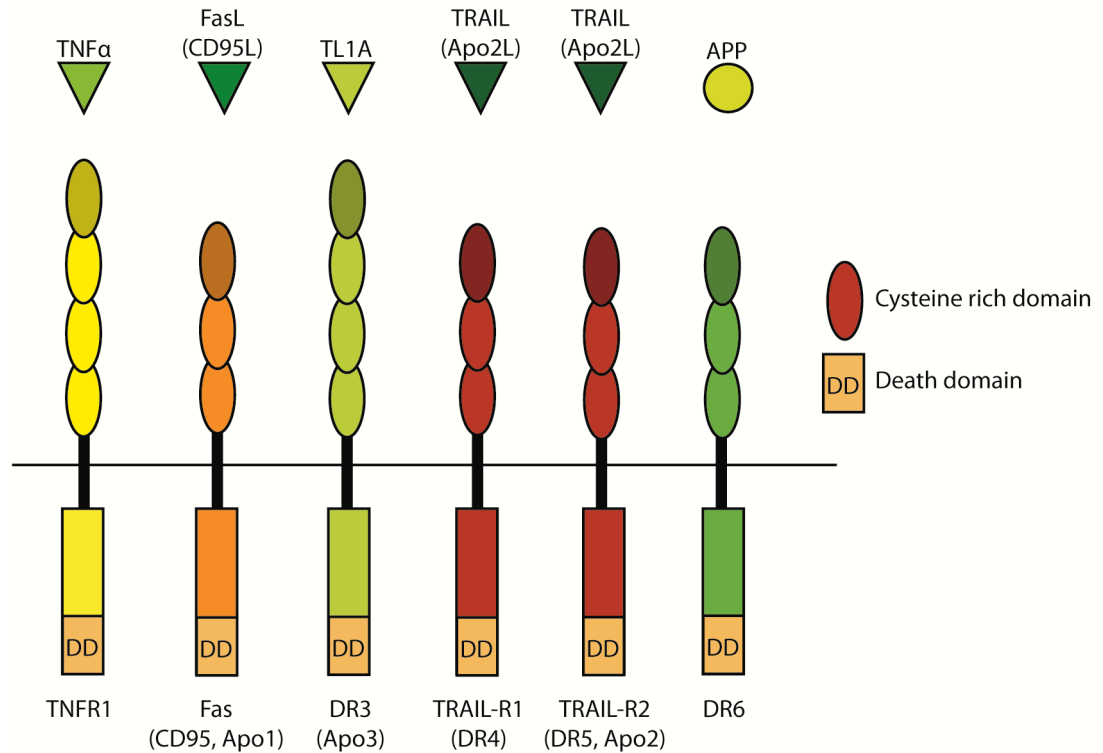
The mitochondrial pathway is induced by various cellular stresses. The pro-apoptotic Bcl-2 proteins Bad, PUMA and NOXA senses cellular stresses and activate BAX and BAK, leading to the permeabilisation of the outer mitochondrial membrane. Permeabilisation leads to the release of cyt c and SMAC to the cytosol. Bcl-2 and BCL-X<sub>L</sub> negatively regulate the mitochondrial permeabilisation. SMAC promotes apoptosis by inhibiting IAPs. Cyt c together with Apaf-1 and

pro-caspase-9 form an apoptosome, facilitating the activation of caspase-9. In turn, caspase-9 cleaves and activates effector caspases such as caspase-3 and -7, finally leading to apoptosis. *Abbreviations: Apaf-1: Apoptotic protease activating factor-1, Cyt c: Cytochrome c, IAP: Inhibitors of Apoptosis, SMAC: second mitochondria-derived activator of caspases*

### **1.3 The death receptor pathway**

The death receptor pathway, also known as the extrinsic pathway, is initiated by the binding of an extracellular death ligand to a death receptor on the cell surface. To date, six death receptors have been identified; Tumour necrosis factor receptor 1 (TNF-R1)[11], Fas receptor (Fas)[12, 13], two TNF related apoptosis inducing ligand (TRAIL) receptors (TRAIL-R1 and TRAIL-R2) [14-18] and the less potent death inducers, Death receptor-3 and -6 (DR3, DR6) [19, 20]. A defining characteristic of the receptors are extracellular domains containing several cysteine rich domains (CRD) that determine ligand specificity. Additionally, the receptors all share a homologous interaction domain of approximately 80-amino acids in the C-terminal cytoplasmic tail, called a death domain (DD). The death receptors bind death-promoting ligands belonging to the TNF superfamily of ligands, such as TNF $\alpha$ , FasL and TRAIL (Figure 2). The ligands are mainly expressed as type-II transmembrane proteins, with a C-terminal extracellular tail, a transmembrane region and an intracellular N-terminal domain. Proteolytic cleavage by metalloproteases can in some cases lead to the release of a soluble ligand. No ligand belonging to the TNF superfamily has been found to bind DR6 so far. Recently the  $\beta$ -amyloid precursor protein was identified as a DR6 ligand; however this interaction does not lead to classical apoptotic downstream signalling [21].





**Figure 2. The death receptors and their ligands**

Six death receptors have been identified to date; TNF-R1, Fas, DR3, TRAIL-R1, TRAIL-R2, and DR6. They all share a homologous death domain in the cytoplasmic tail and extracellular ligand binding CRDs. The receptors bind ligands belonging to the TNF superfamily, except DR6 that binds APP. *Abbreviations: APP:  $\beta$ -amyloid precursor protein, CRD: Cysteine-rich domain, DD: Death domain, DR: Death receptor, FasL: Fas ligand, TL1A: TNF-like ligand 1A, TNF $\alpha$ : Tumour necrosis factor  $\alpha$ , TNF-R1: Tumour necrosis factor receptor 1, TRAIL-R: TNF related apoptosis inducing ligand receptor*

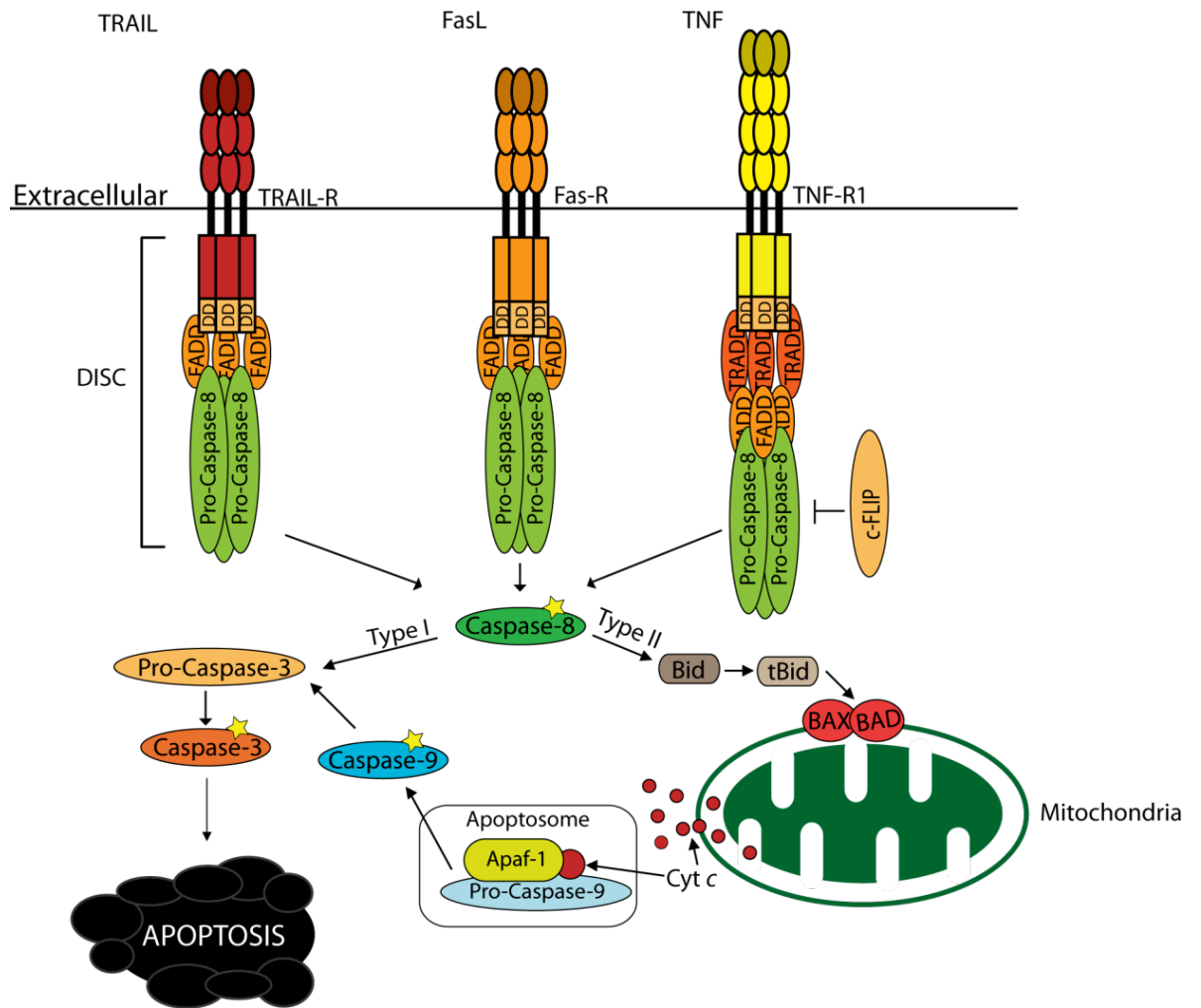
Upon ligation, the death receptors and the ligand bind each other in a trimeric conformation. Ligand binding leads to a conformational change in the DD of the receptor enabling the recruitment of the adaptor protein, Fas associated death domain (FADD), to the intracellular death domain of the receptors [22, 23]. FADD interacts with pro-caspases-8 molecules via a death effector domain (DED) in both proteins, leading to the formation of a complex called the death inducing signalling complex (DISC) (Figure 3). TNFR1 and DR3 require the binding of an additional adaptor molecule, Tumour necrosis factor receptor type 1-associated death domain protein (TRADD), prior to the FADD and caspase-8 recruitment. Assembly of the DISC leads to the cleavage and activation of caspase-8 and further downstream death signalling. Pro-caspase-

10 also binds FADD and is activated at the DISC, however the significance of caspase-10 binding is not fully understood. Caspase-10 has been reported to be unable to functionally substitute caspase-8 [24].

The mechanisms by which the initiator caspases are activated are still being debated and several mechanisms have been proposed. According to the “induced proximity model”, initiator caspases have a low intrinsic activity and are therefore capable of trans-activate each other when the caspase molecules are in close proximity at a high local concentration [25]. A second modified model called the “proximity-induced dimerisation model” has also been suggested. According to this model, the accumulation of initiator caspases in the DISC promotes dimerisation of the caspases leading to activation, suggesting that while cleavage is not required for the activation, it may stabilise the active form [26]. In the most recent model, the “induced conformation model”, initiator caspases are suggested to be activated due to a conformational change in the active site following binding to adaptor protein complexes [27]. Others however claim that both dimerisation and cleavage is required for caspase-8 activation [28]. Ultimately, activation of caspases-8 leads to cleavage and activation of caspase-3 and other effector caspases, causing the cell to undergo apoptosis.

Cells can be classified as type I or type II by the mechanisms in which apoptosis is induced. In type II cells the formation of the DISC is slow and less active caspase-8 appears to be generated. The death signal in type II cells therefore requires amplification by the activation of the mitochondrial pathway. The bridge between the two pathways is achieved through caspase-8 dependent cleavage of the pro-apoptotic Bcl-2 protein Bid to a truncated form (tBid), which triggers the mitochondrial pathway by promoting MOMP and cyt c release. In type I cells, the apoptotic signalling amplitude via the death receptors is sufficient to induce apoptosis independently of the mitochondria [29, 30] (Figure 3).

There are several modulators of death receptor signalling. The cellular FLICE-like inhibitory protein (c-FLIP) is highly homologous to caspase-8 and contains a death effector domain. c-FLIP is capable of FADD binding but lacks protease activity. There are three known splice forms of c-FLIP; c-FLIP long (c-FLIP<sub>L</sub>) and two shorter forms, c-FLIP short (c-FLIP<sub>S</sub>) and c-FLIP Raji (c-FLIP<sub>R</sub>). Binding of c-FLIP<sub>S/R</sub> to the DISC hinders the activation of caspase-8, thereby inhibiting the transduction of the death signal [31-33]. The function of c-FLIP<sub>L</sub> is however more controversial. While some reports propose that c-FLIP<sub>L</sub> primarily exhibits an anti-apoptotic role [34], others suggest that physiological expression levels actually facilitate the activation of caspase-8 and that c-FLIP<sub>L</sub> only acts as an inhibitor at high ectopic protein levels [35].



**Figure 3. The death receptor pathway**

The death receptor pathway is initiated by death promoting ligands binding to transmembrane death receptors. Following ligation, the adaptor protein FADD binds the death domain of the death receptor, directly or indirectly via TRADD, followed by the recruitment of inactive pro-caspase-8 molecules. Together they form a complex called a DISC, facilitating the auto-activation of caspase-8 which in turn activates caspase-3 and subsequent cell death. Type I cells can induce apoptosis independent of the mitochondria, whereas type II cells require amplification of the death signal via the mitochondrial pathway. Caspase-8 dependent cleavage of the Bcl-2 protein, Bid, functions as the link between the two pathways. *Abbreviations: Apaf-1: Apoptotic protease activating factor 1, c-FLIP: Cellular FLICE-like inhibitory protein, DD: Death domain, DISC: Death inducing signalling complex, FADD: Fas associated death domain, Fas-R/L: Fas receptor/ligand, tBid, truncated Bid, TNF: Tumour necrosis factor, TRADD: Tumour necrosis factor receptor type 1-associated death domain protein, TRAIL: TNF related apoptosis inducing ligand*

#### **1.4 TRAIL receptor signalling**

The tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is one of the more recently identified death-inducing ligands of the TNF cytokine family [36, 37]. Interestingly, TRAIL promotes apoptosis in many cancer cells, whereas most normal cells are resistant. The TRAIL receptors are therefore considered as a promising target for cancer therapy [38].

TRAIL is a 281 amino acid, type 2 transmembrane bound glycoprotein, expressed in various tissues; in particular spleen, prostate, lung and varies of immune cells, such as macrophages, natural killer cells and dendritic cells, depending on cell stimulation [36, 37, 39]. The extracellular C-terminal part of TRAIL can be cleaved off; however the activity of the soluble form is generally lower. TRAIL forms homotrimers, with a zinc-binding site required for the structure and function of the trimeric complex. Trimerisation of TRAIL increases the biological activity compared to its monomeric form [37, 40]. TRAIL ligand binds three TRAIL receptors, leading to the formation of a trimeric receptor complex [41, 42].

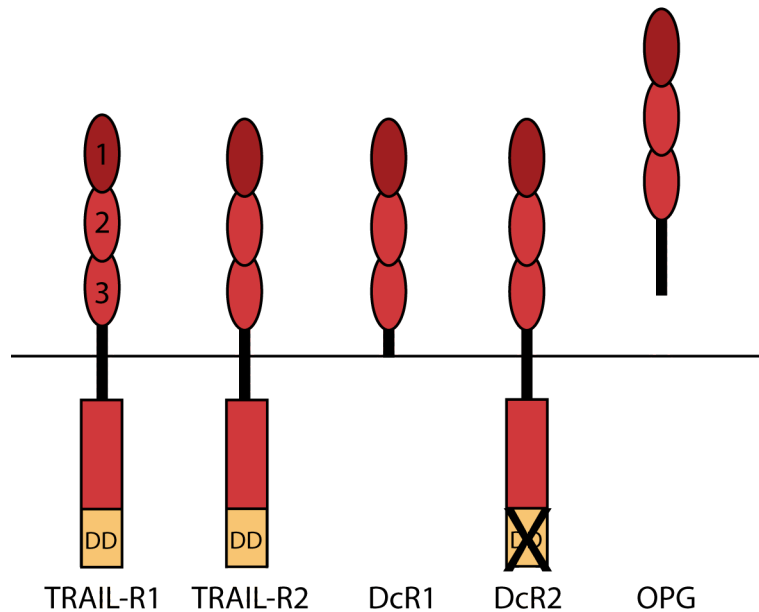
The biological role of TRAIL is still not fully understood. TRAIL has been shown to be essential for the regulation of apoptosis in the immune system *in vivo*. It has also been suggested to have both immunosuppressive and immunoregulatory functions by eliminating auto-reactive immune cells (reviewed by [43]). Several studies also suggest that TRAIL is important for the immune surveillance of developing tumours and metastasis, thereby functioning as a tumour suppressor [44]. Gene-expression studies of 368 human breast tumour samples found that down regulation of TRAIL correlates with brain metastasis [45]. In addition, studies on TRAIL receptor (mDR5) knockout mice demonstrated that while the mice develop normally, tumour formation and metastasis are increased in several tissues such as skin and mammary gland [46]. It has also been shown that TRAIL-deficient mice have defective thymocyte apoptosis, which is responsible for negative selection, leading to an increased susceptibility to autoimmune diseases [47]. There is

however much yet to be understood with regard to TRAIL function, as a contrasting, study reported that loss of mDR5 in the intestine or thymus did not increase tumour formation [48] .

### **1.5 The TRAIL receptors**

To date, five TRAIL binding receptors have been identified in human. Two of them are death receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), capable of inducing apoptosis [14-18]. They are both transmembrane receptors with an extracellular cysteine-rich ligand binding domain and a functional intracellular death domain. The death domain of TRAIL-R1 shares 30% homology to the corresponding domains in TNFR-1 and DR3, but only 19% homology is shared with the death domain of Fas [14].

Two other receptors binding TRAIL, Decoy receptor 1 (DcR1) and Decoy receptor 2 (DcR2), both have a highly similar extracellular ligand-binding domain to that of TRAIL-R1 and -R2 [15, 18]. However, the receptors are unable to induce apoptosis due to the absence of a functional death domain. DcR1 is anchored to the membrane via glycosylphosphatidylinositol (GPI) and is completely lacking a death domain, whereas DcR2 contains a truncated death domain (Figure 4). The decoy receptors can inhibit TRAIL induced apoptosis either by sequestering TRAIL or by forming heteromeric receptor complexes with TRAIL-R1 or -R2, preventing DISC formation [49-51]. The fifth TRAIL binding receptor is the secreted Osteoprotegerin (OPG), which binds TRAIL with low affinity. The significance of this interaction *in vivo* is however not yet fully understood, although it has been suggested that OPG might sequester TRAIL and thereby block apoptosis [52, 53].

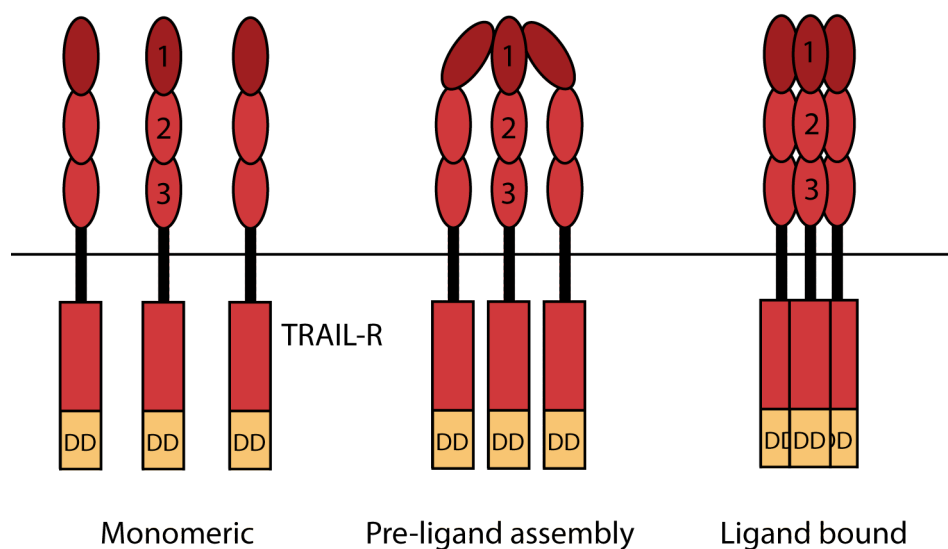


**Figure 4. TRAIL binding receptors**

TRAIL binds five receptors: two death receptors (TRAIL-R1 and -2), with functional death domains capable of inducing apoptosis, two decoy receptors (DcR1 and -2) with a missing or non-functional death domain respectively and the soluble receptor OPG at a lower affinity. *Abbreviations: DcR: Decoy receptor, DD: Death domain, OPG: Osteoprotegerin, TRAIL-R: TNF related apoptosis inducing ligand receptor*

The TRAIL receptors are widely expressed, with particularly high expression in liver, spleen and thyroid [39]. As previously mentioned, in contrast to humans, mice only exhibit one TRAIL receptor called mDR5, which is homologous to both human TRAIL-R1 and R2 [54]. It has yet to be explained why humans have evolved to express two TRAIL receptors and whether there is a significant difference in their signalling cascades. TRAIL-R2 shares 58% overall homology to TRAIL-R1 with the greatest homology found in the intracellular death domain. Distinct roles of the two receptors have not yet been shown, although by using receptor selective agonists it has been shown that certain cell types depend more or less on one of the receptors [55]. For example, TRAIL induced apoptosis of chronic lymphocytic leukemic (CLL) and pancreatic tumour cells been shown to be mediated primarily via TRAIL-R1, even in the presence of functional TRAIL-R2 [55, 56], whereas others report that TRAIL-R2 is a more potent inducer of apoptosis in hepatocytes and lung cancer cell lines [57].

Similar to other death receptors, the extracellular domain of the TRAIL receptors consists of three cysteine rich domains (CRD). The ligand-binding site is within CRD2 and 3 of TRAIL-R2 [41] (Figure 5). It was first believed that the death receptors existed as monomers in the absence of ligand. However several studies have shown that TNFR1 and Fas can self-associate before ligand binding in the membrane independently of ligand binding. The receptors interact via pre-ligand-binding assembly domains (PLAD) in CRD1 (Figure 5) [58, 59]. A PLAD interaction has also been suggested for the TRAIL receptors and Dcr2 has been proposed to form heteromeric pre-ligand complexes together with TRAIL-R1 and -R2, thereby preventing proper DISC formation [59, 60]. The significance of the pre-ligand trimerisation of the TRAIL receptors needs to be further investigated and the interaction has not yet been confirmed with endogenously expressed protein.



**Figure 5. TRAIL ligand binding and pre-ligand trimerisation model**

A PLAD interaction, independent of ligand binding, via the CRD1 of the receptor has been suggested for the TRAIL receptors. TRAIL interacts with CRD2 and CRD3.

*Abbreviations: CRD: Cysteine rich domain, DD: Death domain, PLAD: Pre-ligand-binding assembly domain,*



### **1.5.1 Alternative signalling mediated through TRAIL receptors**

The death receptors were initially thought to only induce apoptotic signalling. However it is now known that TRAIL binding can also mediate signalling via alternative signalling pathways. The death receptors have been shown to regulate processes such as proliferation, inflammation and migration [61-63]. TRAIL can for example stimulate the inhibitor of  $\kappa$ B kinase (IKK) [64], c-Jun N-terminal kinase (JNK), and p38 MAPK signalling cascades. Activation of IKK leads to the degradation of inhibitor of  $\kappa$ B (I- $\kappa$ B) resulting in Nuclear factor  $\kappa$ B (Nf $\kappa$ B) activation and transcription of genes involved in immune function, apoptosis and inflammation. The biological significance of TRAIL induced Nf $\kappa$ B activation is however not fully understood. Some report that Nf $\kappa$ B activation can promote invasiveness, survival and proliferation in some TRAIL resistant cell lines [62], whereas others claim that Nf $\kappa$ B signalling can increase TRAIL sensitivity in human  $\beta$ -cells [65].

The alternative signalling pathways are activated by the formation of a secondary signalling complex (Complex II) subsequent to the DISC assembly. Complex II consists of FADD, caspase-8, receptor-interacting protein 1 (RIP1), TNF receptor-associated factor 2 (TRAF2) and IKK $\gamma$  [66].

Further understanding of mechanisms directing death receptor signalling towards death or non-death actions is needed in order to better understand resistance mechanisms and how to overcome them.

### **1.5.2 TRAIL resistance mechanisms and cancer therapeutic potential**

TNF $\alpha$  and FasL efficiently induce apoptosis in cancer cells. However, the use of the ligands in cancer treatment is not feasible due to severe toxicity after systemic administration. Treatment with Fas or TNF $\alpha$  caused extensive apoptosis of hepatocytes leading to liver failure in mice [67]. On the other hand, recombinant TRAIL has been shown to be generally safe in *in vivo* studies in mice [68] as well as in cancer patients assessed during clinical trials [69]. Antagonistic antibodies targeting the TRAIL receptors, such as mapatumumab

and lexatumumab, have also been tolerated well in patients. However, concerns were raised with certain tagged recombinant TRAIL and anti-TRAIL receptor mAbs developed, where the drug had toxic effect on hepatocytes [70, 71].

Due to the low toxicity of TRAIL in normal cells, TRAIL signalling is still considered as a promising target for cancer therapy. Even though cancer cells are significantly more sensitive to TRAIL-induced apoptosis, many cancer cells develop resistance. Innate and required resistance is thus an evolving problem and it is becoming more evident that TRAIL receptor targeting alone as monotherapy might not be sufficient. TRAIL treatment together with a wide range of pharmaceutical agents, such as cisplatin (DNA crosslinker), or Gefitinib (EGFR inhibitor), have been shown to effectively increase the success rate of the treatment [72, 73]. The development of resistance against TRAIL has been intensively studied and several mechanisms have been proposed. However the significance of the different mechanisms varies to a large extent between cell types. Further understanding of the resistance mechanisms and how to overcome them is required in order to develop effective cancer therapeutics.

Loss of cell surface expression of the TRAIL-receptors, due to constitutive endocytosis, has been correlated to TRAIL resistance and could be a potential biomarker for TRAIL sensitivity [74]. Additionally, TRAIL induced c-Cbl mediated ubiquitinylation of the TRAIL receptors leading to degradation of the receptors via proteasomal and lysosomal pathways has been suggested to correlate with early phase TRAIL resistance [75].

Loss of function mutations in TRAIL-R1 and TRAIL-R2 have been identified in human tumours such as breast carcinoma and non-Hodgkin's lymphoma. Most of the mutations identified were in the intracellular death domain important for FADD binding and thereby potentially disturbing DISC formation [76, 77]. Epigenetic silencing, particularly of TRAIL-R1, has been found in up to 70% of glioblastomas [78] and 30% of ovarian carcinomas [79].

Furthermore, downregulation of caspase-8 or overexpression of c-FLIP, which both result in lowering the caspase-8/c-FLIP ratio can also contribute to lower sensitivity to TRAIL [80]. Alteration in the levels or balance between pro- and anti-apoptotic Bcl-2 protein can cause TRAIL resistance in type II cells which require activation of the mitochondrial pathway [81].

Post-translational modifications of the TRAIL receptors have also shown to be important for the sensitivity to TRAIL. *O*-glycosylation of TRAIL-R1 and TRAIL-R2 promotes ligand induced receptor clustering and the formation of the DISC and further downstream signalling. High mRNA expression of *O*-glycosyltransferases, such as *N*-acetyl galactosamine transferase 14 (GALNT14) and *O*-glycan processing enzymes such as fucosyltransferase 3 (FUT3) have been correlated with an increased sensitivity for TRAIL-induced apoptosis in several tumours [82]. Additionally a putative N-glycosylation site has been detected in TRAIL-R1 and inhibition of N-glycosylation impairs the clustering of TRAIL-R1 but not TRAIL-R2 [83].

### **1.5.3 TRAIL receptor dynamics and trafficking**

Little is known about the trafficking and spatiotemporal regulation of the TRAIL receptors. The dynamics of Fas and TNFR1 have however been studied more in detail. Fas and TNFR1 require ligand dependent internalisation to endosomal compartments for the formation of the DISC, caspase-8 activation and a full apoptotic response [84, 85]. Inhibition of endocytosis in type I cells leads to the inhibition of Fas induced apoptosis, instead leading to survival signalling via MAPK and NF- $\kappa$ B signalling pathways [85]. Fas is internalised via clathrin mediated apoptosis. Knockdown of adaptor protein 2 (AP2), an important adaptor protein, or the heavy chain of clathrin resulted in a lower cell surface expression of Fas and decreased apoptosis [85].

In contrast to Fas and TNFR1, the TRAIL receptors do not require internalisation for the formation of the DISC and induction of apoptosis. Inhibition of endocytosis rather promotes apoptotic signalling via the TRAIL receptors [86]. TRAIL-DISC complexes can be formed without internalisation as

the DISC is formed at 4°C and after inhibition of endocytosis by hypertonic medium or overexpression of dominant negative forms of AP180 or dynamin [86]. TRAIL and its receptors are however rapidly internalised in a concentration- and time-dependent manner after ligand binding, although the biological significance of this is not yet understood [86]. Both clathrin dependent and clathrin-independent pathways have been suggested for the internalisation of the TRAIL receptors [86].

Inhibition of clathrin mediated apoptosis using the drug Chlorpromazine or knockdown of AP2 or Clathrin resulted in a restoration of cell surface expression of the TRAIL receptors and increased sensitivity to TRAIL induced apoptosis. In contrast, inhibition of raft/caveola mediated apoptosis, using the drug filipin III did not restore surface expression or affect the apoptotic response [74]. Others have shown that dynamin inactivation inhibits TRAIL-R2 endocytosis in HELA cells and thereby claim that clathrin mediated endocytosis is required [87]. However, dynamin is involved in both clathrin mediated endocytosis and other routes of endocytosis, so these results do not conclude that other endocytosis pathways might be involved. Endocytosis of TRAIL-R1 is mediated via a sorting signal (EAQC<sup>337</sup>LL) in the intracellular domain. Similar signals are present in many other receptors and have been implicated in mediating the rapid internalization of proteins and targeting to the endosomal-lysosomal compartments. TRAIL-R1 with a mutated sorting signal (EAQC<sup>337</sup>AA) was almost exclusively expressed on the cell surface and the sensitivity to TRAIL was increased [74].

In addition to internalisation, TRAIL signalling is also regulated by the localisation of the receptors in lipid raft micro-domains in the plasma membrane. The role of lipid raft in death receptor signalling is discussed in detail in the next paragraph.

## ***1.6 The role of lipid rafts in death receptor signalling***

### **1.6.1 Definition of lipid raft**

Lipid rafts are defined as dynamic microdomains within cellular membranes enriched with sphingolipids and cholesterol and resistant to detergent extraction. The rafts function as a signalling platform for targeted protein traffic and are involved in endocytosis via flotillin and caveolar-dependent pathways [88]. There are two different types of lipid rafts, the non-invaginated rafts called planar rafts and tube-like invaginations called caveola. In contrast to planar lipid rafts, the morphology of the caveola can be clearly visualised using electron microscopy. The reported size of lipid rafts varies to a large extent, although a recent consensus was made, considering lipid rafts to be between 10–200 nm in diameter [89].

Although lipid rafts have been extensively studied, their biological relevance and even their very existence is still a matter of debate. The doubts are mainly due to limitations and lack of assays to isolate and study lipid rafts. Lipid rafts are considered to be highly dynamic structures and depending on the assay used, variations of protein composition in the raft fraction are observed which further contributes to the controversy [89]. However, a variety of proteins such as receptor tyrosine kinases, adaptor proteins and G-protein coupled receptors are preferentially compartmentalised in lipid rafts and caveola and it is suggested that protein localisation in rafts regulates or promotes signalling events and act as an important signalling platform [90].

Caveolin proteins are key scaffolding proteins important for the formation and function of the caveola. Three isoforms of caveolin have been identified (caveolin 1, 2 and 3). Caveolin-1 and 2 are co-expressed in many cell types, whereas caveolin 3 is mainly expressed in muscle cells [91]. Caveolin-1 forms homo and hetero-oligomers (~15 molecules) with caveolin-2 in the endoplasmic reticulum and in the Golgi and are thereafter trafficked to the cell surface where they form the higher molecular mass complexes involved in caveola formation. Caveolin-1 expression alone has been shown to be sufficient

for caveola formation and the role of caveolin-2 in this process is less understood. Caveolin-1 consists of a central hydrophobic region inserted in inner membrane of the plasma membrane with both C- and N-terminus exposed to the cytoplasm. The C-terminal is important for the oligomerisation, whereas the N-terminal contains a caveolin scaffolding domain (CSD) required for caveolin dimerisation and functions as an interaction domain with several signalling proteins that containing caveolin-binding domains (CBD) (reviewed in [92]).

The family of flotillins are also crucial for the formation of lipid rafts and they interact with actin, thereby linking the rafts to the cytoskeleton of the cell. The cytoskeleton is important for the mobility and distribution of the lipid rafts (reviewed in [93]). Endocytosis can occur via four different raft dependent routes. There are dynamin dependent and independent pathways. Furthermore the dynamin dependent pathway can be divided into Cav1 dependent or independent routes (reviewed by [94]). The Cav1 and dynamin independent endocytosis pathway requires Flotillin-1 and Cdc42 [95].

### **1.6.2 Lipid rafts and death receptor signalling**

Studies have shown that death receptor signalling is dependent on the localisation of death receptors in the plasma membrane. Compartmentalisation of Fas and the TRAIL receptors in lipid rafts have been shown to promote receptor clustering and DISC formation, subsequently promoting apoptotic signalling [96, 97]. It has also been suggested that whereas TRAIL receptors located in lipid rafts mediate apoptotic signalling, receptors in non-rafts promote alternative signalling pathways, such as NfκB signalling [98]. Treatment with several drugs, such as epirubicin, oxaliplatin and quercetin results in a translocation of the TRAIL receptors to lipid rafts and increased receptor clustering leading to increased sensitivity to TRAIL [99-101]. Little is known regarding the mechanisms promoting compartmentalisation of lipid rafts, although the translocation of the death receptors to the rafts appears to be ligand dependent at least in some cell types. In human T-cells, low levels of Fas were detected in lipid rafts in non-treated cells followed by a significant

translocation of the receptor following FasL treatment. Moreover, treatment with a lipid raft disrupting drug, methyl-beta-cyclodextrin, inhibited the apoptotic response, further supporting the importance of the lipid rafts for efficient downstream cell death signalling [102]. Similarly, experiments performed using the human lung carcinoma cell line, NCI-H460, showed TRAIL-R1 and -R2 to be primarily localized to lipid rafts after treatment with TRAIL [103]. In contrast, TRAIL-R2 is reported to be constitutively localised in lipid rafts in the Jurkat cell lines TIB152 and TIB153, [104].

S-Palmitoylation functions as a key signal for the FasL dependent redistribution of Fas to lipid raft domains [105]. S-palmitoylation is a reversible post-translational lipid modification, attaching a 16-carbon fatty acid to cysteine residues known to target several signalling proteins, such as Ras GTPases, to lipid rafts (reviewed in [106]). TRAIL-R1 is also palmitoylated, promoting lipid raft localisation and receptor oligomerisation. Palmitoylation of TRAIL-R2 and TNFR1 has not been observed [107].

In addition to the palmitoylation of the Fas receptor, a lysine rich region (LRR) in the cytoplasmic region adjacent to the transmembrane domain has been identified as a lipid raft targeting signal necessary for Fas-mediated cell death. The LRR region consists of four or five lysines surrounding the palmitoylation site. TRAIL-R2 and TNFR1 also have a region similar to the LRR of the Fas receptor, which may contribute to the lipid raft localisation [108].

Further studies investigating the dynamics of lipid rafts and TRAIL receptor signalling is however required. Since the location of the TRAIL receptors in lipid rafts have been shown to be able to sensitise cells to TRAIL, further understanding of the lipid raft dynamics may contribute to development of new cancer therapy. In this thesis, the Activated Cdc42-associated kinase 1 (Ack1) is identified as a protein promoting TRAIL induced apoptosis by inducing lipid raft localisation of the TRAIL receptors.

## **1.7 Activated Cdc42-associated kinase 1**

Activated Cdc42-associated kinase 1 (Ack1) also known as tyrosine kinase, non-receptor 2 (Tnk2) has been described to regulate a variety of cellular events. Some of which are summarised below and might be linked to the novel role of Ack1 in TRAIL signalling reported in this thesis.

Ack1 was first cloned in 1993 from a human hippocampal expression library, identified as an interacting partner with the activated form of the Rho-GTPase Cdc42 [109]. The human Ack1 gene is located at chromosome 3q29 [110], a region which have been associated with hematologic malignancies and [111] adrenocortical carcinomas [112].

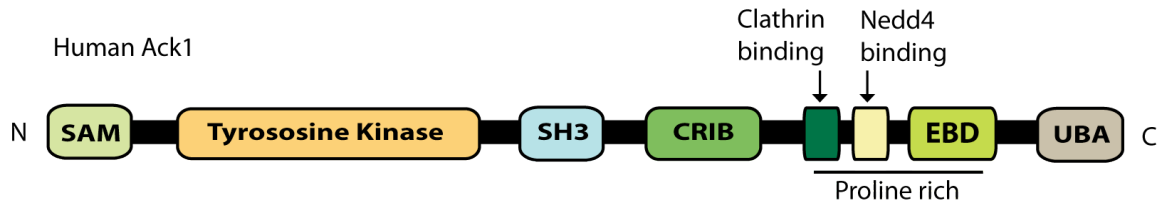
Ack1 can be activated by several external stimuli such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin and integrin mediated adhesion. A pool of Ack1 protein is constitutively phosphorylated, even after prolonged starvation [110].

### **1.7.1 Structure of Ack1**

Ack1 is a 120 kDa tyrosine kinase containing a series of domains and binding regions (Figure 6). The protein consists of a N-terminal sterile  $\alpha$ -motif (SAM) - like domain [110], followed by a tyrosine kinase domain, a Src-homology 3 (SH3) domain, a Cdc42/Rac interactive binding (CRIB) domain and a conserved C-terminal proline-rich region homologous to the ErbB receptor inhibitor Mitogen inducible gene 6 (Mig6), functioning as a generic RTK-binding domain [113]. The SAM-like domain has been suggested to function as a membrane-targeting signal, thereby regulating the sub-cellular localisation of Ack1 [110]. The tyrosine residue, Y284, found in the activation loop of the kinase has been identified as the primary autophosphorylation site in Ack1. A mutation of the Y284 residue leads to a significant decrease in protein phosphorylation [114]. Ack1 also interacts with the adaptor Grb2 mediating the recruitment of receptor tyrosine kinases such as Axl, leukocyte tyrosine kinase (LTK) and the anaplastic lymphoma kinase (ALK) [113].



Ack1 contains several protein-protein interaction domains and a series of interaction partners and phosphorylation targets have been identified, however the significance of many of the interactions is not known.



**Figure 6. Domain structure of Ack1**

Ack1 is a non-receptor tyrosine kinase consisting of an N-terminal SAM-like domain followed by a tyrosine kinase domain, a SH3 domain, CRIB domain, a C-terminal proline-rich region homologous to the ErbB receptor inhibitor Mig6 and a UBA domain in the C-terminus. *Abbreviations: CRIB: Cdc42/Rac interactive binding, EBD: EGFR binding domain, Mig6: Mitogen inducible gene 6, SAM: sterile  $\alpha$ -motif, SH3: Src-homology 3, UBA: Ubiquitin associated*

### 1.7.2 Role of Ack1 *in vivo*

Ack1 is ubiquitously expressed, with the highest expression in brain, spleen and thymus [110]. Amplification of the ACK1 gene and/or overexpression of Ack1 mRNA have been identified in a variety of human primary tumours and been correlated with a poor prognosis [115]. The overexpression and gain of copy number were most evident in late stage primary tumours and metastatic tumours but were uncommon in early stage tumours, suggesting that cancer cells acquiring overexpression of Ack1 are more prone to metastasise. The highest frequency of ACK1 amplification/overexpression was detected in lung, ovarian and prostate primary tumours [115]. *in vivo* experiments in mice, with implantation of cancer cells overexpressing Ack1, showed that Ack1 contributes to a significantly increased tumour burden in the lungs [115]. A transgenic mouse model overexpressing an activated form of Ack1 (L487F) exclusively in the prostate, develop prostatic epithelial neoplasia [116]. Ack1 has been suggested to promote prostate tumour progression via an ErbB2/ErbB3 dependent mechanism, by phosphorylation and activation of the Androgen receptor (AR) [117] Ack1 has also been reported to phosphorylate the putative tumour suppressor, WW domain containing oxidoreductase

(Wwox), and thereby targeting it for poly-ubiquitinylation and degradation [118].

#### 1.7.2.1 The role of Ack1 in ErbB receptor signalling

Ack1 is implicated in the regulation of Epidermal growth factor receptor (EGFR) signalling however conflicting results have been published and the details regarding the role of Ack1 have yet to be resolved. Ack1 interacts with the EGFR receptor through a conserved EGFR-binding domain (EBD) in the proline rich region of the protein. The interaction between Ack1 and EGFR is dependent on the tyrosine kinase activity of EGFR, but independent of activation status of Ack1 [119]. One study proposes that Ack1 induces EGFR degradation via the putative ubiquitin association (Uba) domain in the C-terminus. Knockdown of Ack1 or overexpression of an Uba-domain defective mutant in HEK293 cells, inhibited EGF-induced internalisation of EGFR [119]. Others report that both overexpression and siRNA mediated knockdown of Ack1 leads to decreased internalisation rate of EGF. In addition, an increased recycling of EGFR in Ack1 knockdown cells has been reported, suggesting that Ack1 promotes endosomal sorting of the receptor to the endosomes for degradation [120]. In contrast to this finding, an additional study suggests that Ack1 promotes cell surface expression of EGFR in breast epithelial cells [121]. In *C. elegans*, the related protein A Ras-regulating Kinase 1 (Ark-1) is functioning as negative regulator of Let-23 signalling, the *C. elegans* homologue of EGFR [122]. However the role of Ack1 in mammalian ErbB signalling is not conclusive.

#### 1.7.2.2 Ack1 and endocytosis

Additionally, Ack1 also interacts with several proteins involved in endocytosis and trafficking. Two clathrin heavy chain (CHC) binding domains have been identified [123, 124] and Ack1 also interacts with adaptor protein 2 (AP2), Amphiphysins-1 and 2 and sorting nexin 9 (SNX 9) all components of vesicle dynamics. The role of Ack1 in clathrin mediated endocytosis is however not fully understood. Overexpression of ACK1 leads to disturbed clathrin distribution, although moderate ectopic expression stimulated the endocytosis

of the transferrin receptor, known to be internalised via clathrin mediated endocytosis [124]. In addition it has also been suggested that the phosphorylation of Ack1 is dependent on localisation of the protein at clathrin coated pits and Cdc42 activation [123].

#### 1.7.2.3 Ack1 is activated by Cdc42

Ack1 is a downstream effector of the Rho GTPase, Cell division control protein 42 (Cdc42) [109] Cdc42 is involved in a variety of cellular functions, such as cell morphogenesis, migration and cell cycle progression (see 1.8). Ack1 mediates cell migration via a Cdc42 dependent mechanism, by phosphorylating the adaptor protein p130<sup>cas</sup>, crucial for integrin-mediated signalling pathways promoting cell migration [125]. Moreover, Ack1 can phosphorylate Wiskott-Aldrich syndrome protein (WASP) and thereby promote its actin remodelling activity. In this thesis, a novel role of Ack1 in death receptor signalling has been discovered, possibly via a Cdc42 dependent mechanism.

### 1.7.3 Activation and regulation of Ack1

It has been suggested that Ack1 is an auto-inhibited kinase, regulated by intramolecular interactions. Two models have been proposed, one where the EGFR binding domain in the proline-rich region binds to the kinase domain [126] and secondly an interaction between the SH3 domain and the proline-rich region, potentially forming an auto-inhibited state [110]. However, these two models have recently been questioned by Manser et al., 2011. In their work, point mutations in the SH3 and ErbB binding region did not activate Ack1, as has previously been reported. In contrast, they suggest that the oncogenic tyrosine kinase Src is required for Ack1 activation by phosphorylation of Tyr<sup>284</sup> in the activation loop, functioning as the key regulator [127].

The regulation of the turnover of Ack1 has yet to be fully understood; both proteasomal and lysosomal degradation have been proposed. Two E3 ubiquitin ligases; neural precursor cell expressed and developmentally down regulated 4-1 and -2 (Nedd4-1 and Nedd4-2) have been shown to interact with Ack1, via a conserved PPXY-containing region in the proline rich domain of Ack1, and

target the protein for degradation [128, 129]. Nedd4-2 and Ack1 have been shown to co-localise in clathrin-rich vesicles and Nedd4-2 is proposed to down-regulate Ack1 by ubiquitinylation leading to targeting and degradation in the proteasome. The proteasome inhibitor MG132 can inhibit this degradation. Kinase activity of Ack1 is required for the interaction with Nedd4-2 [128]. In contrast, another study reported that Ack1 levels are regulated by the Nedd4-1 ligase and not Nedd4-2, even though both interact with Ack1. They also claim that Ack1 is degraded in the lysosome and not the proteasome, since lysosomal inhibitors inhibited degradation whereas proteasomal inhibitors did not [129].

#### **1.7.4 The role of Ack1 in apoptosis**

A role for Ack1 in cell death has only been described once where Ack1 was classified as survival kinase in an RNAi screen targeting all known kinases. Apoptosis was induced by cytotoxic drugs (paclitaxel, cisplatin, or etoposide), activating the mitochondrial pathway. Cell death was assessed using an enzyme-linked immunosorbent assay (ELISA) against histone-associated DNA fragments. Knockdown of Ack1 induced  $\geq 2.5$  times more apoptosis compared to control cells, leading it to be classified as a survival kinase. However, the phenotype was not further characterised and no molecular mechanism was described [130].

In this thesis, a novel role of Ack1 in death receptor signalling is presented, showing that Ack1 is crucial for TRAIL induced apoptosis. We also show that Cdc42 is required for TRAIL apoptosis, possibly by activating Ack1. Below is a summary of Cdc42 function and regulation.

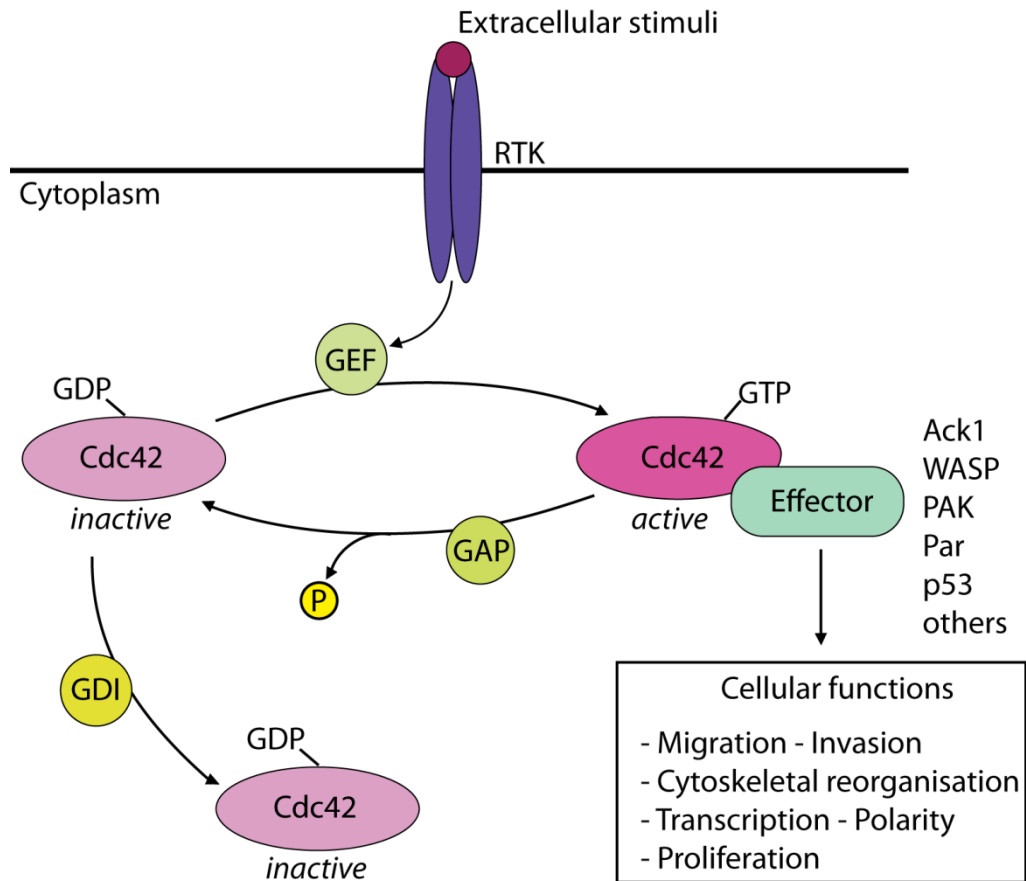
### **1.8 *Cdc42 mediated cell signalling***

Cdc42 belongs to the Rho family of small GTPases together with Rho and Rac. Cdc42 functions as a molecular switch, reverting from a GDP inactive state to a GTP-bound active state in response to multiple stimuli such as growth factors, cytokines and cell-cell interactions. In its active form, Cdc42 interacts with a series of effector proteins thereby activating signalling pathways involved in a variety of cellular processes such as migration, invasion,

reorganisation of the cytoskeleton, endocytosis, vesicle trafficking, transcription, cell polarity and cell cycle progression (Figure 7). The activity of Cdc42 is strictly regulated by three groups of regulators; guanine nucleotide exchange factors (GEFs) facilitate the hydrolysis from GDP to GTP, GTPase activating proteins (GAPs) increase the GTPase activity of Cdc42 leading to inactivation, and guanine nucleotide-dissociation inhibitors (GDI) sequester Cdc42 from the cellular membrane, preventing its activation (reviewed in [131]).

Cdc42 promotes actin polymerisation and remodelling via N-WASP and Arp2/3, important for regulating the formation of microspikes and filopodia [132-135]. Cdc42 also modulates the formation of cell polarity by regulating vesicle trafficking in the cytoplasm towards the apical or basolateral side of the cell [136]. Cdc42 interacts with proteins regulating endocytosis and Cdc42 also mediate traffic between ER and Golgi. Inhibiting Cdc42 alters membrane traffic in polarized epithelial cells, affecting traffic via both the trans-Golgi network and the recycling between the plasma membrane and endosomes [136-138].

Furthermore, Cdc42 regulates cell-cell junctions by regulating the endocytosis and trafficking of junctional proteins, although by which mechanism is still controversial. Whereas some suggest that active Cdc42 is required for stable E-cadherin adhesion at adherens junctions by actin cytoskeleton remodelling [139, 140], others report that Cdc42 promotes ubiquitination and degradation of E-cadherin, subsequently leading to the dissolution of adherens junctions [141]. Lack of Cdc42 also leads to mislocalisation of junctional proteins such as  $\alpha$ - and  $\beta$ -catenin in drosophila [142] and degradation of  $\beta$ -catenin in mice, causing impairment of cell-cell contacts [143]. Cell-cell junctions are discussed further in 1.9.



**Figure 7. Cdc42 regulation and signalling**

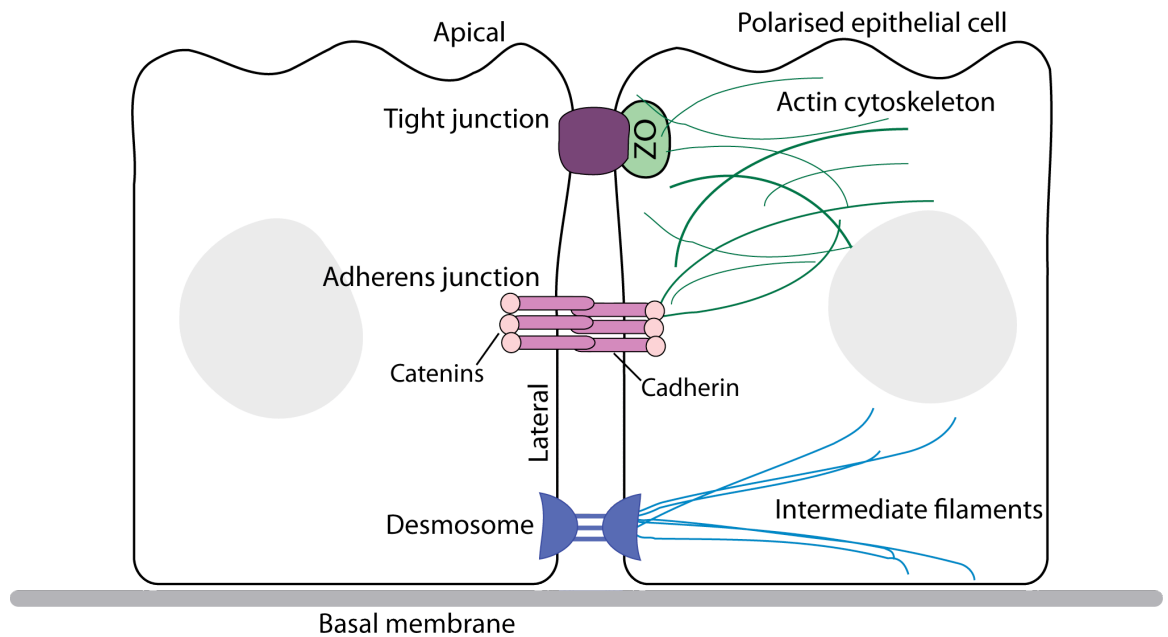
The small GTPase, Cdc42, switches from an inactive (GDP-bound) to an active (GTP-bound) state. Active Cdc42 can interact with multiple effector proteins, such as Ack1 and WASP, leading to the activation of downstream signalling pathways regulating a variety of cellular processes. Cdc42 is tightly regulated by three groups of regulatory proteins; GEFs, that mediate the hydrolysis of GDP to GTP, GAPs, that increase the GTPase activity of Cdc42 leading to inactivation and GDIs, that sequester Cdc42 in the cytosol preventing its activation. *Abbreviations: Ack1: Activated Cdc42-associated kinase 1, Cdc42: Cell division control protein 42 homolog, GAP: GTPase activating proteins, GDI, guanine nucleotide-dissociation inhibitors, GEF: Guanine nucleotide exchange factor, PAK: p21-activated protein kinase, RTK, Receptor tyrosine kinase, WASP: Wiskott–Aldrich syndrome protein*

### 1.9 Epithelial cell-cell junctions

While elucidating the role of Ack1 in TRAIL induced apoptosis, we also discovered that Ack1 is required for cell-cell junction stability or formation.

Epithelial cells are joined together by a series of intercellular junctions at the lateral side of polarized cells. There are four different types of junctions; tight junctions (TJs), adherens junctions (AJs), gap junctions (GJs) and desmosomes. The TJs are the most apical junctions in epithelial cells, functioning as a strong barrier preventing leakage of molecules between the apical and basal side of the cells. They are often considered as the border between the apical and the basolateral side of a cell and have been suggested to play a role in polarity. Gap-junctions function as a passage of small water-soluble molecules between cells and are important for intercellular communication (reviewed in [144]). ZO-1 is a commonly used TJ junction marker and was the first TJ-associated protein discovered [145] (Figure 8).

AJs and desmosomes provide adherent strength between cells and are linked to the actin cytoskeleton and intermediate filaments respectively. The desmosomes provide extra adherent strength and are more abundant in cells undergoing mechanical stress such as epidermis and cardiac muscle. Both AJs and desmosomes are highly calcium dependent and share a similar structure. Cadherins, mainly E-Cadherin in adherens junctions and the desmosomal cadherins, including desmocollins (Dscs) and desmogleins (Dsgs), forms the physical link between the cells. The cadherins in turn interact with armadillo proteins bound to catenins such as  $\beta$ -,  $\alpha$ -catenin and desmosomal catenins, such as  $\gamma$ -catenin, which function as the link to the cytoskeleton (Figure 8). While cell-cell junctions were previously considered to only function as structural bonds between cells, it is now clear that junctional complexes can also function as signalling platforms. The cadherin–catenin complex can interact with several other proteins, including signalling proteins and cell surface receptors, thereby functioning as a center for protein–protein interactions [146].



**Figure 8. Cell-cell junctions in epithelial cells.**

The tight junctions are the most apical junctions in epithelial cells and function as a barrier between the apical and basal side and are also important cell signalling platforms. The adherens junctions provide adherent strength between cells. Both tight junction and adherens junctions are linked to the actin cytoskeleton. The desmosomes provide adherent strength and are connected to the intermediate filaments. *Abbreviations: ZO: Zona occludens*

Loss of cell-cell junctions and adhesion is a characteristic of epithelial to mesenchymal transition (EMT). During EMT, epithelial cells acquire more mesenchymal-like morphology promoting migration and invasiveness of the tumour cells through the basal membrane and the extracellular matrix. Junctional proteins typical for epithelial cell junctions such as  $\beta$ -catenin and E-cadherin are downregulated, whereas mesenchymal proteins like, N-cadherin and vimentin are upregulated (reviewed in [147]).

In this thesis, a novel role of Ack1 in TRAIL receptor signalling and cell-cell junction stability and formation is presented.



## **Research Aim**

TRAIL receptor signalling has been extensively studied the last 15 years, however further mechanistic insight of how the receptors are regulated and trafficked is needed. Innate and acquired resistance to TRAIL has evolved to be a big hurdle in the use of recombinant TRAIL and TRAIL receptor agonist as cancer therapy.

In this work, the non-receptor tyrosine kinase Ack1 is reported to be crucial for TRAIL induced apoptosis in human epithelial cells. The aim with this thesis is to investigate the molecular mechanisms by which Ack1 regulates TRAIL induced apoptosis. This will be investigated specifically by cell biology and biochemical methods assessing the receptor cell surface expression, subcellular localisation of the TRAIL receptors, oligomerisation and DISC formation.

## **Chapter 2 - Materials and methods**

### **3.1 Cell culture**

#### **3.1.1 Cell line maintenance**

The non-tumourigenic human mammary epithelial cell line; MCF10A, obtained from American Type Culture Collection (ATCC), was cultured in DMEM/F12 (Invitrogen) supplemented with 5% horse serum (v/v) (Invitrogen), 20 ng/ml EGF (Milenyi Biotech), 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin and 10 µg/ml insulin (all from Sigma). HELA (human cervical carcinoma cells) (from Wolfson Institute for Biomedical Research (WIBR) cell repository) was cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (v/v) (PAA clone, PAA Laboratories Ltd.). SW480 cells (human colon carcinoma cells) (WIBR cell repository) was cultured in Leibovitz's L15 media (Invitrogen) supplemented with 10% FBS. The human lung carcinoma cell line, NCI-H460 (WIBR Cell Repository), was cultured in RPMI-1460 with 10% FBS. All cell cultures were cultivated at 37°C with 5% CO<sub>2</sub>, except SW480 cells which was cultured in 100% air. Cells were routinely passaged three times per week by trypsinisation, using 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) (Invitrogen) in PBS with 0.02% EDTA.

#### **3.1.2 Cryopreservation of cultured cell lines**

All cell lines used were cryopreserved in liquid nitrogen for long-term storage. Cells were washed in PBS, trypsinised and thereafter centrifuged at 74 x g for 3 minutes (min) in an Eppendorf 5804 centrifuge. The cells were resuspended in freezing media (70% FBS, 20% DMEM and 10% dimethyl sulfoxide (DMSO) (Sigma), (v/v)) at a concentration of approximately 1-2 x 10<sup>6</sup> cells/ml and aliquoted in cryovials (Corning). The vials were deposited in a 5100 Cryo 1°C freezing container (Nalgene) with isopropanol and placed at -80°C, allowing a cooling rate of 1°C per min. The vials were transferred to liquid nitrogen for long-term storage after 24 hours (h) storage at -80°C.

### **3.1.3 Thawing of cryopreserved cell lines**

A cryovial with cells was quickly thawed in a 37°C water bath and transferred to a tube containing 4 ml pre-warmed (37°C) complete cell culture media. Cells were centrifuged for 3 min at 74 x g and resuspended in cell culture media, suitable for the specific cell line, and plated in a 10 cm cell culture dish (Corning).

### **3.2 RNA interference transfection**

MCF10A, SW480 and HELA cells were transfected with siRNA at a final concentration of 12 nM with Lipofectamine RNAi MAX (Invitrogen). NCI-H460 cells were transfected twice, 5h apart, with 30 nM siRNA per transfection. The media was replaced prior to the second transfection. For the siRNA used see Table 1. Cells were trypsinised and seeded minutes before transfection, so they would reach a density of approximately 60% following day. siRNA and Lipofectamine RNAiMAX were diluted in Opti-MEM (Invitrogen) in separate tubes and thereafter combined, mixed gently, and incubated at room temperature (RT) for 15 min. The transfection mixture was slowly added to the cells dropwise and the media was changed the day after transfection. The volumes used for different cell culture dish formats are outlined in Table 2. Experiments were performed 48h (MCF10A, SW480 and HELA) or 72 h (NCI-H460) post-transfection. Due to a slightly reduced proliferation rate following Ack1 knockdown (see 4.1.5), 25% more cells were transfected with the Ack1 siRNA oligo compared to control cells, in order for the cells to obtain comparable confluence at the start of the experiment. The Ack1(b) oligo was used for the knockdown of Ack1 unless other stated.

**Table 1. siRNA sequences**

siRNA used for knockdown of Ack1 and Cdc42. Non-targeting siRNA was used as a control.

Target gene (name of oligo)	Sequence (sense 5' → 3')	siRNA ID#	Manufacturer
Ack1(a)	GGAUGAGGAUGACUUUGAGTT	103419	Applied Biosystems
Ack1(b)	GGUGUUCAGUGGAAAGCGATT	s19850	Applied Biosystems
Cdc42 (a)	n/a	D-005057-01	Calbiochem
Cdc42 (b)	n/a	D-005057-02	Calbiochem
Cdc42 (c)	n/a	D-005057-04	Calbiochem
Non-targeting (Ctrl)	n/a	AM4636	Applied Biosystems

**Table 2. Volumes used for siRNA transfection**

MCF10A, SW480, and HELA cells were transfected with 12 nM siRNA. NCI-H460 cells were transfected twice with 30 nM siRNA per transfection, 5h apart. The siRNA stock concentration was 100  $\mu$ M. The cell culture dish/plate format used was depending on the assay performed. The total volume stands for transfection mixture together with cell culture media per dish or per well in a 12-well plate.

Format	siRNA (12nM/30nM)	Opti-MEM	Lipofectamine RNAiMax	Total volume
12-well plate	0.15 $\mu$ l/0.38 $\mu$ l	2 x 75 $\mu$ l	1.1 $\mu$ l	1 ml
3.5 cm dish	0.3 $\mu$ l/0.75 $\mu$ l	2 x 150 $\mu$ l	2.2 $\mu$ l	2ml
6 cm dish	0.6 $\mu$ l/1.5 $\mu$ l	2 x 400 $\mu$ l	5.8 $\mu$ l	4ml
10 cm dish	1.2 $\mu$ l/3 $\mu$ l	2 x 1 ml	15 $\mu$ l	8 ml
15 cm dish	3.9 $\mu$ l/9.75 $\mu$ l	2 x 2.6 ml	39 $\mu$ l	25 ml

### 3.3 Plasmid DNA transfection

H460 cells were transfected with full length (wtAck1) or kinase dead Ack1 (KD-Ack1(K158A)) cloned in a pcDNA-DEST40 expression vector (See 3.5.2 and 3.5.3) using JetPRIME® transfection reagent (Polyplus Transfection™). H460 cells were seeded in a 12-well plate to reach a confluency of approximately 60% at the time of transfection. Following concentrations and volumes were used for one well in a 12-well plate: 0.8 $\mu$ g plasmid DNA was diluted in 75 $\mu$ l JetPRIME buffer and vortexed for 10s. 2 $\mu$ l JetPRIME reagent was added and the

solution was vortexed for 10s. The solution was incubated at RT for 10min and thereafter added drop wise to the cells. The medium was replaced after 16h and experiments were performed 48h post-transfection.

### **3.4 DNA purification**

#### **3.4.1 Selective antibiotics**

Final concentrations of selective antibiotics used for bacterial cultures were; 100 µg/ml ampicillin, 100 µg/ml spectinomycin, 50 µg/ml kanamycin and 50 µg/ml chloramphenicol. 1000x stock solutions of ampicillin, kanamycin and spectinomycin were prepared by dissolving the antibiotics in H<sub>2</sub>O followed by filter sterilisation. Chloramphenicol was dissolved in ethanol, 500x concentrated.

#### **3.4.2 Mini-prep DNA purification**

Mini-prep DNA purification was performed using the GeneJET™ plasmid miniprep kit (Fermentas) according to the suppliers' recommendation. A single colony from a selective agar plate was inoculated in 3 ml Luria broth (LB) media (20g/l LB broth (Sigma) in H<sub>2</sub>O) containing the appropriate selective antibiotics and incubated at 37°C for approximately 6-8h, shaking at 200-250 revolutions per minute (rpm) (Inova 44 incubator shaker, New Brunswick Scientific). The bacterial culture was harvested by centrifugation for 5 min at 6000 x g at RT in a table top centrifuge (Heraeus Biofuge Pico). The pelleted bacteria was resuspended in 250 µl resuspension solution followed by addition of 250 µl lysis solution and 350 µl neutralisation solution. After the addition of each buffer, the sample was mixed by inverting the tube 4-6 times. The sample was centrifuged for 10 min at 16,000 x g. The supernatant was transferred to a GeneJET™ spin column and centrifuged for 1 min at 16,000 x g followed by two washes with 0.5 ml wash solution. In order to elute the DNA, 50 µl elution buffer was added to the column and centrifuged for 1 min. The DNA concentration was determined using a Nano drop ND1000 spectrophotometer at 260 nm (Thermo Scientific).

### **3.4.3 Midi-prep DNA purification**

Midi-prep DNA purification was performed using the QIAprep spin midiprep kit (Qiagen). A single colony was inoculated in 5 ml LB media supplemented with appropriate selective antibiotics and cultured in a shaking incubator, at 200-250 rpm, for 6-8h at 37°C. The culture was diluted 1:500 into 50 ml selective LB media and cultured o/n at 37°C shaking at 200-250 rpm. The bacteria was harvested by centrifugation at 6000 x g for 15 min at 4°C (Beckman Avanti J20, Beckman Coulter™) and resuspended in 4 ml buffer P1. In order to lyse the cells, 4 ml buffer P2 was added and incubated for 15 min at RT. The DNA was precipitated by the addition of 4 ml buffer P3 and incubated on ice for 15 min. The sample was centrifuged for 30 min at 10,000 x g. A Qiagen-tip 100 column was equilibrated by adding 4 ml buffer QBT to the column and emptied by gravity flow. The supernatant was added to the column followed by two washes with 10 ml buffer QC. The DNA was eluted from the column by the addition of 5 ml buffer QF and thereafter precipitated with 3.5 ml isopropanol and centrifuged immediately at 3220 x g for 1h at 4°C (Eppendorf centrifuge 5810R). The supernatant was carefully removed and the pellet was washed in 2 ml 70% ethanol and centrifuged again for 1h at 3220 x g at 4°C. The pellet was air dried for 5 min and thereafter dissolved in 200 µl EB buffer. The DNA concentration was determined using a Nano drop ND1000 spectrophotometer at 260 nm.

### **3.4.4 Glycerol stock**

Bacterial glycerol stocks for long term storage was prepared in cryovials by combining 70% glycerol with 30% bacterial culture (v/v) and stored at -80°C.

## ***3.5 Molecular cloning***

### **3.5.1 Preparation of LB agar plates**

LB agar (Sigma) was dissolved in dH<sub>2</sub>O at a concentration of 35g/l and sterilised by autoclaving. The LB agar was left to cool down to 55°C and appropriate selective antibiotics were added to the solution (see 3.4.1.) The

agar was poured into 10 cm Petri dishes, approximately 20 ml/dish and let to solidify for 30 min, thereafter stored at 4°C.

### 3.5.2 Isolation of Ack1 cDNA

Full-length human ACK1 open reading frame (ORF) cDNA, cloned into the, Gateway® compatible, entry vector pENTR223.1 (Cat. #HOC19832, Accession#NM\_005781) was purchased from GeneCopoeia. The plasmid was transformed into one shot TOP10® (Invitrogen) competent *Escherichia coli* (*E.coli*) cells for propagation according to the suppliers' recommendations and plated on a spectinomycin selective agar plate and incubated o/n at 37°C. Plasmid DNA was isolated using the GeneJET™ plasmid mini prep kit as previously described in 3.4.2. The accurate size of the insert was confirmed by restriction analysis. DNA sequencing of the complete Ack1 cDNA, performed by the WIBR Scientific Support Services, confirmed the correct sequence of the insert. For sequencing primers used see Table 3.

**Table 3. Ack1 sequencing primers**

Sequencing primers used for nucleotide sequencing of Ack1 cDNA. The N-terminal region of Ack1 was sequenced using a primer targeting the polylinker in the pENTR223.1 vector prior to the Ack1 insert.

Name	Starts at nucleotide	Primer sequence 5'→3'
1	Polylinker in pENTR223.1	CGTTGCAACAAATTGATGAGC
2	121	GTCAAGAATGAGGACCTGGAG
3	495	GAGCCAGCCAGAAGCCATG
4	922	GCGACACCTGGATGTTCCG
5	1390	CACACAGGGCATGGCGAC
6	2348	CCCTGTCCCCTCAAGGCTC
7	2769	CTCCACCAACAACAGCAACCC

### 3.5.3 Site-directed mutagenesis

Site-directed mutagenesis is a technique for making point mutations, deletion or insertion of amino acids at particular sites of the DNA. This is done by several temperature cycling steps. The first step is to denature a double stranded (dsDNA) vector containing an insert of the gene to be altered. This is followed by annealing of synthetic oligonucleotide primers, containing the desired mutation. The primers, each complementary to the opposite strand, are then extended by DNA polymerase, and a plasmid with the incorporated mutation in the gene is generated. The parental DNA strand, without the mutation, is thereafter degraded by endonucleases, for example with the *Dpn* I endonuclease, specifically cleaving methylated and hemimethylated DNA (dsDNA with only one methylated strand).

Prior to sub-cloning of Ack1 into an expression vector, site-directed mutagenesis was performed. A stop codon was inserted in the C-terminal end of the cDNA, to be able to express non-tagged protein. Additionally, non-sense mutations in the Ack1b siRNA binding region was made, making it possible to ectopically overexpress the Ack1 construct in a siRNA knockdown background (See Table 1 for siRNA targeting sequence). Furthermore, several point mutants were generated, either affecting the kinase activity or protein-protein interaction sites in Ack1 (Table 4).

**Table 4. Ack1 constructs generated by site directed mutagenesis**

Name of construct	Mutation/alteration
<b>Ack1(stop)</b>	Insertion of stop codon
<b>siR-Ack1</b>	siRNA resistant, Ack1b siRNA binding region, no amino acid change
<b>Kd-Ack1</b>	Point mutation in active loop, kinase dead
<b>Ca-Ack1</b>	Point mutation resembling constitutively active form
<b>CRIB-Ack1</b>	Point mutation abolishing binding to Cdc42
<b>Clath-Ack1</b>	Point mutations abolishing binding to Clathrin heavy chain

Site-directed mutagenesis was performed using the QuikChange® XL Site-Directed Mutagenesis kit from Stratagene. Specifically, a sample reaction containing 2.5 µl 10x reaction buffer, 5 ng pENTR223.1 vector with wtAck1



inserted (see 3.5.2), 50 ng of forward and reverse oligonucleotide primer, 0.5  $\mu$ l dNTP mix, 1.5  $\mu$ l QuikSolution, ddH<sub>2</sub>O to a final volume of 25  $\mu$ l and 0.5  $\mu$ l of PfuTurbo DNA polymerase was made. The oligonucleotide primers were obtained from Invitrogen (Table 5). Each sample reaction was cycled on a S1000™ Thermal Cycler (BioRad) as outlined in Table 6. Following temperature cycling, the reaction was cooled to  $\leq 37^{\circ}\text{C}$ . In order to digest the non-mutated parental template, 0.5  $\mu$ l *Dpn* I restriction enzyme (Stratagene) was added followed by incubation for 1h at  $37^{\circ}\text{C}$ . The DNA was thereafter transformed into XL 10-Gold *E.coli* cells for propagation.

### **3.5.3.1 XL 10-Gold transformation**

For each transformation reaction, aliquots of 45  $\mu$ l of XL10-Gold® ultracompetent *E.coli* cells (Stratagene) were transferred to pre-chilled 14-ml BD Falcon polypropylene round- bottom tubes (BD Biosciences). To the cells, 2  $\mu$ l of  $\beta$ - mercaptoethanol mix (Stratagene) was added and incubated on ice for 10 min. Following incubation, 2  $\mu$ l of the *Dpn* I treated DNA, from the site-directed mutagenesis (see 3.5.3) was combined with the cells and incubated on ice for 30 min. The transformation reaction was thereafter heat-shocked in a  $42^{\circ}\text{C}$  water bath for 30 s, incubated on ice for 2 min and 0.5 ml preheated ( $42^{\circ}\text{C}$ ) NZY broth media (1 % NZ amine (casein hydrolysate), 0.5 % yeast extract, 0.5 % NaCl, (Stratagene)) was added to each reaction. The transformations were incubated for 1h at  $37^{\circ}\text{C}$  in a shaking incubator (225-250 rpm). Finally, 250  $\mu$ l of the transformation was added to a pre-warmed agar plate with suitable selective antibiotic and incubated over night at  $37^{\circ}\text{C}$ . Following day, single colonies from the plate were inoculated in 3 ml LB media with 50  $\mu\text{g/ml}$  ampicillin and cultured for 6-8h at  $37^{\circ}\text{C}$ . Plasmid DNA was isolated using the GeneJET™ miniprep kit as described in 3.4.2. Sequence analysis by WIBR Scientific Support Services confirmed that the mutagenesis was successful. For Ack1 sequencing primers used see Table 3.

**Table 5. Oligonucleotide primer sequences for site-directed mutagenesis**

Oligonucleotide primer sequences used for site directed mutagenesis of Ack1. Underlined nucleotides were mutated or inserted. Grey highlighted residues represent the amino acid codon changed. *Abbreviations*, Ack1, Activated Cdc42-associated kinase 1, KD: Kinase dead, Ca: constitutively active, Clath: Clathrin, CRIB: Cdc42/Rac interactive binding domain, siR: siRNA resistant

Mutant	Mutation	Primer sequence (5'→3')
Ack1(stop)	3114 +TAA	CACCACAAGCGCT <u>TAA</u> TCAGGCCTCATGGGC (Forward) GCCCATGAGGCCTGATTAGCGCTTGTGGTG (Reverse)
siR-Ack1	cagt240-243tttc; a246g; g249a (no amino acid change)	GGATGAGTAAGGTGTTT <u>TTTCGGG</u> AA <u>ACG</u> ACTGGAGGC TGAG (Forward) CTCAGCCTCCAGTCGTTTCCCGAAAAACACCTTACTCATCC (Reverse)
KD-Ack1	K158A a472g ; a473c	GGTGAGTGTGGCTGTG <u>GCC</u> GCCTGAAGC (Forward) GCTTCAGGCACGCCACAGCCACACTCACC (Reverse)
Ca-Ack1	L487F c1459t;g1461t	GGATTGACGAACTGTAT <u>TTT</u> GGAACCCCATGGACCC (Forward) GGGTCCATGGGGTTTCCAAAATACAGTTCGTCAATCC (Reverse)
CRIB-Ack1	H464D/H467A c1390g; c1399g ; a1400c	GCTTCATC <u>GAC</u> ACAGGG <u>GCT</u> GGCGACAGTG (Forward) CACTGTCGCCAGCCCCTGTGTCGATGAAGC (Reverse)
Clat-Ack1	L571A a1711g;t1712c  SVE496AAA	(L571A) GCTGAGGTCACGCTC <u>CCC</u> GACTTCGGTGAGG (Forward) CCTCACCGAAGTCGGCGAGCGTGACCTCAGC (Reverse)  (SVE496AAA) CCCCCGACGCCGCCAGCGTGGAAC (Forward) AGTTCCACGCTGGCGGCGTCGGGGG (Reverse)

**Table 6. Temperature cycling parameters for site-directed mutagenesis of Ack1**

Segment	Cycles	Temperature	Duration
1	1	95°C	3 minutes
2	18	95°C	50 seconds
		60°C	50 seconds
		68°C	11 minutes
3	1	68°C	7 minutes

#### **3.5.4 LR-recombination reaction for subcloning of Ack1 cDNA into mammalian expression vector**

A Gateway® LR Recombination Reaction was performed in order to combine the Ack1 cDNA, contained in the entry vector (pENTR223.1), with a mammalian expression vector (destination vector) (pcDNA/DEST40) (Invitrogen) by homologous recombination, generating an expression clone.

Specifically, a LR-reaction containing 100 ng pENTR223.1, with wild type or mutated Ack1 inserted (see 3.5.3), 150 ng pcDNA/DEST40 vector and 6.5 µl TE-Buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) to a final volume of 8 µl was made. To the sample, 2 µl of LR Clonase™ II enzyme mix (Invitrogen) was added and incubated at 25°C for 2 h. To terminate the reaction, 1 µl Proteinase K solution (Invitrogen) was added and incubated for 10 min at 37°C. The construct was thereafter transformed into one shot TOP10® competent cells and DNA was purified as previously described in 3.4.2 or 3.4.3.

#### **3.5.5 Generation of StrepII-tagged Ack1**

An N-terminal StrepII tag was inserted to the wtAck1 expression construct by ligation of annealed StrepII oligos via a HindIII restriction site in the pcDNA/DEST40 vector.

##### **3.5.5.1 Insertion of HindIII restriction site**

A HindIII restriction site was inserted in the pcDNA/DEST40 vector by site directed mutagenesis as described in 3.5.3. The restriction site was placed as

close as possible to the ATG start codon of the wtAck1 sequence. For the mutagenesis primer used see Table 7.

### 3.5.5.2 5' Phosphorylation and annealing of complimentary oligos

Complimentary oligos with the StrepII coding sequence flanking the N-terminal of Ack1 was designed (Table 7B). The primers were 5' phosphorylated in order to increase ligation efficiency. A reaction containing 100 pmol of each forward and reverse primer, 2.5 µl 10x PNK buffer, 2.5 µl ATP (stock 10 mM), 0.5 µl (5 U) T4 polynucleotide kinase (T4 PNK) (All from New England BioLabs) and 18.5 µl H<sub>2</sub>O to a total volume of 25 µl was made. The reaction was incubated at 37°C for 30 min. The T4 PNK was thereafter inactivated by incubation at 70°C for 10 min. For the annealing of the complimentary oligos, 15 µl (60 pmol) of each 5'phosphorylated oligos was mixed with 3 µl NEB buffer 2 and thereafter incubated for 5 min at 95°C, 5 min at 70°C, 5 min at 37°C and then cooled down to RT.

**Table 7. Primer sequences for the insertion of StrepII tag in pcDNA/DEST40.**

**A.** Oligonucleotide primer for insertion of a HindIII restriction site in the pcDNA/DEST40 vector by site-directed mutagenesis. **B.** Oligonucleotide primer for insertion of StrepII tag.

	Primer	Primer sequence
<b>A</b>	Insertion of HindIII restriction site	5'CGTCAAGGCCCACCAAGCTTCAGCCAGAGGAGG3' (Forward) 5'CCTCCTCTGGCTGAAGCTTGGTGGGCCTTGACG3' (Reverse)
<b>B</b>	Strep II tag	5'AGCTTACCATGTGGAGCCACCCACAATTTCGAGAAGTCAGCAA 3' (Forward) 5'AGCTTTGCTGACTTCTCGAATTGTGGGTGGCTCCACATGGTA3' (Reverse)

### 3.5.5.3 Digestion & Ligation

The wtAck1-pcDNA/DEST40 vector, with the HindIII restriction site, was digested with HindIII in order to linearise the vector. Specifically, 500 ng plasmid DNA was incubated with 2 µl NEB 2 buffer and 0.5 µl HindIII restriction enzyme (New England BioLabs) in H<sub>2</sub>O at a final volume of 20 µl for 1 h at 37°C. The digested vector was thereafter incubated with 0.5 µl Antarctic

Phosphatase (New England BioLabs) at 37°C for 1h. The phosphatase was then inactivated at 65°C for 10 min. The DNA was purified with a PCR purification kit according to the suppliers' protocol (Qiagen). The DNA was eluted from the PCR purification column with 30 µl H<sub>2</sub>O. The annealed StrepII primers and the digested pcDNA/DEST40 vector was ligated by incubating 50 ng digested vector with 1 µl annealed oligo, 1 µl T4 ligation buffer, 0.5 µl T4 ligase (all from New England BioLabs) and H<sub>2</sub>O to a final volume of 10 µl. The reaction was incubated at RT for 1 h and 0.5 µl of the ligation reaction was transformed into TOP10® competent cells according to suppliers' recommendations. Single colonies from the transformation were cultured and plasmid DNA mini preps was made as previously described in 3.4.2. Insertion of the correct tag sequence and orientation of the insert was confirmed by sequencing performed by WIBR Scientific Support Services. The forward sequencing primer used started in the CMV promoter of pcDNA/DEST40 (5'TGGGCGGTAGGCGTG3').

### **3.6 Western blotting**

#### **3.6.1 Preparation of samples for western blotting**

Cells were washed with ice-cold PBS once and harvested with 1.5 x SDS-sample buffer (120 mM Tris-HCl pH 6.8, 3 % SDS, 15 % glycerol, 0.03 % bromophenol blue, 75 mM DTT). In order to degrade the genomic DNA, 0.5 U/µl Benzonase (Merk) was added to the lysate and incubated at RT for approximately 15 min prior to 3 min incubation at 95°C. The samples were cooled on ice and directly subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

#### **3.6.2 SDS-PAGE**

Proteins were separated by SDS-PAGE according to size. The separation gel was prepared as described in

Table 8 and was allowed to polymerise for a minimum of 30 min prior to the overlay of the stacking gel. The percentage of gel used (7.5%, 10% or 12.5%) was depending of molecular weight of the protein of interest being resolved. Lower percentage gels better resolve heavier proteins and vice versa. The separation gel was overlaid with a stacking gel. A gel comb with 10 or 15 wells

was placed in the stacking gel directly after pouring it onto the separation gel and the gel was left to polymerise for a minimum of 30min. A 4% stacking gel was used for 10% and lower percentage separation gels, whereas the 6% stacking gel was used for 12.5% gels (Table 9.). The gels were placed in a mini-protein running module and the module was placed in a mini-protean running tank (both from Bio-Rad) filled with SDS-running buffer (15 mM Tris base, 19.6 mM glycine, 1% SDS). The comb was removed from the gel and protein lysates were loaded into the wells. To determine the molecular weight of the proteins the Page Ruler pre-stained protein marker (Fermentas), ranging from 170 kDa to 10 kDa was used as a reference. The electrophoresis was run at 100 V (constant voltage) until the dye front had run out the gel.

**Table 8. Recipe for separation gel used in SDS-PAGE**

Volumes used to cast two SDS-PAGE gels. The percentage of gel used was depending on the molecular weight of the protein of interest being resolved. Low percentage gels resolve larger proteins more efficiently and vice versa. The gel was allowed to polymerise for a minimum of 30 min. *Abbreviations: APS: ammonium persulfate SDS: Sodium dodecyl sulphate, TEMED: N, N, N', N'-tetramethylethylenediamine,*

Percentage	7.5%	10%	12.5%
H <sub>2</sub> O	5.8 ml	4.9 ml	3.9 ml
0.5 M Tris-HCl (pH 8.8)	3.0 ml	3.0 ml	3.0 ml
30% acrylamide/bis-acrylamide	2.95 ml	3.7 ml	4.75 ml
10% SDS	120 µl	120 µl	120 µl
TEMED	16 µl	16 µl	16 µl
25% APS	16 µl	16 µl	16 µl

**Table 9. Recipe for stacking gel used in SDS-PAGE**

Volumes used to cast two SDS-PAGE gels. 4% stacking gels was used for 10% and 7.5% separation gels, whereas a 6% stacking gel was used for 12.5% separation gels. *Abbreviations: APS: ammonium persulfate, SDS: Sodium dodecyl sulphate, TEMED; N, N, N', N'-tetramethylethylenediamine*

Percentage	4%	6%
H <sub>2</sub> O	3.6 ml	3.6 ml
0.5M Tris-HCl (pH 6.8)	630 µl	630 µl
30% Acrylamide/bis-acrylamide	660 µl	1 ml
20% SDS	25 µl	25 µl
TEMED	10 µl	10 µl
25% APS	5 µl	5 µl

### 3.6.3 Immunoblotting and detection

Proteins were transferred onto Hybond™-P PVDF membranes (GE Healthcare) using the Mini Trans-Blot® wet transfer system (Bio-Rad). The PVDF membrane was incubated in methanol, fiber pads and filter paper were soaked in transfer buffer (25 mM Tris-base, 19.6 mM Glycine with 10% methanol added prior to use) and the gel sandwich was thereafter assembled submerged in transfer buffer. The proteins were transferred at 100 V for 60 or 90 min in transfer buffer, depending on the molecular weight of the protein of interest. Proteins larger than 90 kDa were transferred for 90 min.

The membranes were blocked in 4% (w/v) skimmed dry milk or 5% BSA (w/v) dissolved TBS-T, depending on primary antibody used, for 1h at RT. The membranes were incubated with primary antibody diluted in milk or BSA blocking buffer for 3h at RT or o/n at 4°C on a rocker. See Table 10 for primary antibodies used. Membranes were washed three times with TBS-T, for 10 min each, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-mouse or goat anti-rabbit (Jackson Laboratories)) diluted 1:3000 in milk blocking solution for 1h at RT, followed by three more washes with TBS-T. The protein bands were visualised by chemiluminescence using ECL™ or ECL plus™ reagents and Hyperfilm® ECL (all from GE Healthcare). The film was processed using a Compact-X developer (X-ograph imaging systems). GAPDH or  $\alpha$ -tubulin was used as protein loading control.

#### **3.6.4 Stripping and re-probing of PVDF membranes**

The membrane washed in TBS-T twice for 10 min and incubated in stripping buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS and 100 mM of 2-Mercaptoethanol) for 30 min at 56°C. The washing step was repeated and the membrane was blocked in 4% (w/v) skimmed dry milk, dissolved in TBS-T, for 1h at RT followed by primary antibody incubation and all the subsequent steps described in 3.6.3.



**Table 10. Primary antibodies used in western blotting.**

Membranes were blocked and antibodies were diluted in TBS-Tween with 4% dry milk (w/v) unless other stated. *Abbreviations: BSA: Bovine serum albumin, M: Mouse, R: Rabbit*

Targeting protein	Clone	Catalogue number	Host	Manufacturer	Dilution
<b>Ack1</b>	A-11	Sc-28336	M	Santa Cruz Technology	1:200
<b><math>\alpha</math>-Tubulin</b>	-	-	M	Home made	1:10 000
<b><math>\beta</math>-catenin</b>	H-102	Sc-7199	R	Santa Cruz Biotechnology	1:5000
<b>Caspase-8</b>	1-1-37	MAB4708	R	Millipore	1:500
<b>Caveolin-1</b>	Polyclonal	610406	M	BD Transduction Laboratories	1:4000
<b>Cdc42</b>	44	610928	M	BD transduction Laboratories	1:1000
<b>Cleaved Caspase-3 (Asp175)</b>	Polyclonal	9661	R	Cell signaling Technology	1:2000
<b>Cleaved Caspase-8 (Asp391)</b>	18C8	9496	R	Cell signaling Technology	1:2000 Blocking: Milk Antibody: 5 % BSA
<b>FADD</b>	Polyclonal	2782	R	Cell signaling Technology	1:500
<b>Keratin 8/18</b>	5D3	NCL-5D3	M	Novocastra Laboratories Ltd.	1:5000
<b>Na<sup>+</sup>/K<sup>+</sup>ATPase</b>	Polyclonal	3010	R	Cell signaling Technology	1:4000
<b>Phospho-Ack1* (Tyr284)</b>	Polyclonal	09-142	R	Millipore	1:1000
<b>Phospho-Akt (Thr308)</b>	C31E5E	2965	M	Cell signalling Technology	1:2000 BSA
<b>Phospho-EGFR (Tyr1068)</b>	Polyclonal	2234	R	Cell signaling Technology	1:2000 5 % BSA
<b>pNFkB (Ser536)</b>	93H1	3033	R	Cell signalling Technology	1:2000 5 % BSA
<b>Streptavidin-HRP</b>	n/a	FP1047	n/a	Perkin Elmer	1:5000
<b>TRAIL-R1</b>	Polyclonal	1139	R	ProSci Incorporated	1:4000

<b>TRAIL-R2</b>	Polyclonal	2019	R	ProSci Incorporated	1:4000
<b>ZO-1</b>	Polyclonal	40-2300	R	Zymed Laboratories	1:2000

\* Works only on overexpressed protein

### **3.7 Immunocytochemistry**

#### **3.7.1 Collagen coating of coverslips**

Glass cover slips, 13 mm in diameter, thickness #1.5, 0.16 - 0.19 mm (VWR International) was used for all immunocytochemistry (ICH) experiments. The coverslips were sterilised by autoclaving and thereafter coated with 0.01% collagen type I (rat tail, BD Biosciences) (v/v) diluted in 0.02M acetic acid. The coverslips were coated for 2h at 37°C and washed twice with PBS prior seeding of cells.

#### **3.7.2 Immunostaining**

NCI-H460 cells were cultured on collagen I coated coverslips and thereafter fixed with ice-cold methanol for 10 second at RT. The cells were blocked with 5% normal goat serum in TBS-T (TBS (100 mM Tris base pH 7.6, 1.5M NaCl) with 0.1% Tween-20 (v/v)) for 30 min at RT. Primary antibodies were diluted 1:100 in blocking solution and 30 µl antibody solution was added to each coverslip and incubated for 2h at RT. For primary antibodies used see Table 11. The coverslips were briefly washed five times with TBS-T, thereafter incubated with secondary antibody, diluted 1:300 in blocking solution, for 1h at RT in the dark. Secondary antibodies used were donkey-anti-rabbit Cy3 (gift from Taija Makinen, LRI) and donkey-anti-mouse Alexa 488 (Invitrogen). The coverslips were washed an additional five times with TBS-T and stained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma), diluted in PBS, for 5 min at RT in the dark. Finally, the coverslips were washed twice in PBS and mounted with Vectashield H-1000 mounting media (Vector Laboratories,) on a Superfrost® microscope slide (Thermo Scientific). The coverslips were sealed to the microscope slide with nail polish around the edges of the coverslip and let to dry at RT, prior to storage at 4°C. Confocal microscopy acquisitions were acquired at London Research Institute, Lincoln's Inn Fields, Cancer Research UK with the technical assistance of Andres Vicente. The images were acquired

using a Zeiss LSM inverted confocal microscope (Carl Zeiss) at a magnification of 63x using the software LSM 510. Orthogonal views were obtained using the LSM Image Browser software.

**Table 11. Primary antibodies used for immunocytochemistry**

Primary antibodies used for immunocytochemistry staining of NCI-H460 cells. All antibodies were diluted 1:100 in TBS-T (TBS with 0.1 % Tween-20). *Abbreviations: Ack1: Activated Cdc42-associated kinase 1, Na<sup>+</sup>/K<sup>+</sup> ATPase: Sodium-potassium adenosine triphosphatase TRAIL-R1: TNF-related apoptosis-inducing ligand receptor 1, ZO-1: Zona occludens 1*

Targeting protein	Clone	Catalogue number	Host	Manufacturer
<b>Ack1</b>	A-11	Sc-28336	M	Santa Cruz Biotechnology
<b>β-catenin</b>	H-102	Sc-7199	R	Santa Cruz Biotechnology
<b>Caveolin-1</b>	Polyclonal	610406	M	BD Transduction Laboratories
<b>γ-catenin</b>	15F11	Ab12083	M	Abcam
<b>Na<sup>+</sup>/K<sup>+</sup> ATPase α1 subunit</b>	Polyclonal	3010	M	Cell Signaling Technology
<b>TRAIL-R1</b>	Polyclonal	1139	R	ProSci Incorporated
<b>ZO-1</b>	Polyclonal	40-2300	R	Zymed Laboratories

### **3.8 Generation and purification of recombinant TRAIL**

The construct for generating recombinant human TRAIL was a kind gift from Dr Marion Macfarlane, University of Leicester. TRAIL (residues 95-281) was cloned into the expression vector pET28b containing an in-frame N-terminal His-6 and T7 tag.

#### **3.8.1 IPTG Induction**

The pET28b-TRAIL construct was transformed into BL21(DE3)RIL competent *E. coli* cells for propagation according to the suppliers' recommendations. The transformation reaction was spread out on kanamycin containing agar plates and incubated o/n at 37°C. One single colony was inoculated in 20 ml LB-media supplemented with 50 mg/ml kanamycin and 50 mg/ml chloramphenicol and incubated o/n at 37°C, shaking at 250 rpm. The 20 ml culture was thereafter diluted in 1 l LB media supplemented with 50 mg/ml kanamycin and 50 mg/ml

chloramphenicol and cultured at 37°C for 2 h, shaking at 250 rpm. To induce protein expression of the recombinant TRAIL, 1 mM IPTG was added to the 1 l culture and cultured for 3 h at 27°C at 250 rpm. The culture was centrifuged at 3220 x g for 15 min at 4°C (Eppendorf centrifuge 5810R) and the pellet was washed once in ice cold PBS and stored at -80°C until purification. In order to control the induction efficiency, 1 ml aliquots were taken from the non-induced and induced culture, pelleted and resuspended in 50 µl 3 x SDS-sample buffer (240 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 0.06 % bromophenol blue, 150 mM DTT) and subjected to SDS-PAGE. The gel was stained with coomassie brilliant blue (0,1% Coomassie brilliant blue R250 (w/v), 25% 2-propanol (v/v), 10 % acetic acid (v/v)) o/n on a rocker and thereafter destained with (10% (v/v) acetic acid, 40% (v/v) methanol) until the background staining was removed.

### **3.8.2 Purification**

Induced bacterial pellets were lysed in Triton lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton-X, 1 mM phenylmethylsulfonyl fluoride (PMSF)) for 30 min on ice and thereafter sonicated. Lysates were cleared by centrifugation at 3220 x g for 45 min at 4°C and then supplemented with Imidazole (Sigma) at a final concentration of 20 mM, to prevent unspecific binding to the nickel beads. His-TRAIL was thereafter batch purified with 1.5 ml (dry volume) Ni<sup>2+</sup>-NTA agarose beads (Qiagen) for 1.5 h at 4°C on a spinning wheel, followed by three, 10 min, washes with Triton lysis buffer at 4°C. As a control of the purification steps performed, 20 µl aliquots were collected after each centrifugation step and resuspended in 40 µl 3x SDS SB and subjected to SDS-PAGE and coomassie staining as previously described in 3.8.1.

### **3.8.3 Elution**

TRAIL was eluted from the beads by the addition of 1 ml elution buffer (300 mM NaCl, 100 mM Imidazole dissolved in PBS). The beads were incubated with the elution buffer on a spinning wheel for 15 min at 4°C, the beads were centrifuged at 500 x g and the supernatant with the eluted TRAIL was collected.

The elution step was repeated an additional five times, leading to the collection of 4 elution fractions in total. The fractions with the highest and purest concentration of TRAIL were pooled. Aliquots from the eluted fractions were resuspended 1:1 in 3X SDS-Sample buffer and run on a 12 % SDS-PAGE gel as described in 3.6.2. The gel was thereafter stained with coomassie brilliant blue as described in 3.8.1. The fractions with the highest purified yield were pooled and thereafter aliquoted, snap frozen in liquid nitrogen and stored at -80°C. The death inducing activity was assessed by cleaved caspase-8 levels by western blotting.

#### **3.8.4 Biotinylation of recombinant TRAIL**

Recombinant TRAIL were generated and purified as previously described in 3.8.1 and 3.8.2. Prior to elution, the beads were resuspended in 1 ml PBS and labelled with 0.4 mg D-biotinoyl- $\epsilon$ -aminocaproic acid-N-hydroxysuccinimide ester (biotin-7-NHS), dissolved in DMSO (Roche) for 1h at 4°C. Excess labelling solution was quenched by the addition of 2 ml 1 M Tris-HCl (pH 8.0) for an additional 15 min at 4°C. The beads were thereafter washed in lysis buffer and eluted with 100 mM Imidazole as previously described. The incorporation of biotin and the apoptotic inducing activity was assessed by western blotting with a HRP conjugated streptavidin antibody and cleaved caspase-8 levels respectively.

#### ***3.9 Induction of apoptosis via the TRAIL induced pathway***

TRAIL induced apoptosis was induced by treating cells, approximately 70-80% confluent, with 30U/ml recombinant TRAIL for the time points indicated.

#### ***3.10 Induction of apoptosis via the Fas induced pathway***

For activation of the Fas ligand induced pathway, 2  $\mu$ g/ $\mu$ l of the activating anti-FAS antibody, clone C11 (#05-201 Millipore, Billerica, MA, US) was added to cells, approximately 70% confluent and incubated for time points specified.

### ***3.11 Cell cycle profiling with PI staining***

MCF10A cells were trypsinised, washed in PBS and counted using a hemocytometer. Each sample was diluted in 1 ml PBS at a concentration of  $1.5 \times 10^6$  cells/ml. The cells were centrifuged at  $200 \times g$  at RT for 5 min and resuspended in 0.5 ml PBS. The cells were fixed by the addition of 4.5 ml of ice-cold 70% ethanol and were incubated on ice for a minimum of 30 min. After the fixation, the cells were centrifuged at  $400 \times g$  for 5 min and washed in 5 ml PBS followed by another centrifugation step. The cells were resuspended in 0.5 ml PBS and 0.5 ml DNA extraction buffer (0,2 M  $\text{Na}_2\text{HPO}_4$ , 0,004% Triton X-100, pH 7.8) The sample was incubated at RT for 5 min and centrifuged at  $400 \times g$  for 5 min. The supernatant was removed and the cells were resuspended in 1 ml freshly prepared DNA staining solution (50  $\mu\text{g/ml}$  PI, 0.2 mg/ml RNase A (dissolved in 10 mM Sodium acetate (pH 7.4) in PBS) for 30 min in the dark. The cell cycle profile was analysed with flow cytometry (CyAn ADP High-Performance Flow Cytometer) at an excitation of 488 nm. Side scatter and emission at  $>600$  nm was measured. A minimum of 10 000 events/sample was collected and the hypodiploid and diploid DNA peaks was measured. The Summit 4.3 software was used to analyse the data.

### ***3.12 FACS analysis of apoptosis with Annexin V/PI labelling***

MCF10A cells cultured in 6 cm dishes, approximately 80% confluent, were treated with 30U/ml TRAIL for 2h. The cells were thereafter washed in PBS and trypsinised. Extra care was taken to ensure the cells were in single cell suspension. The cells were washed twice in Annexin V binding buffer (10 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM  $\text{MgCl}_2$  and 1.8 mM  $\text{CaCl}_2$ ) and 10  $\mu\text{l}$  Annexin V-FITC (Miltenyi Biotec) was added followed by incubation for 15 min in the dark at room temperature. The cells were washed twice in Annexin V binding buffer and 1 mg/ml of PI solution was added immediately prior to analysis by flow cytometry. The software Summit version 4.3 was used to analyse the data. 10 000 events/sample were collected and three independent experiments were performed.

### ***3.13 FACS analysis of TRAIL receptor cell surface expression***

Cell surface expression of TRAIL-R1 and -R2 was analysed as previously described [148]. MCF10A cells were trypsinised, washed once in cell culture media and counted using a hemocytometer. The cells were diluted in 1 ml medium/sample at a concentration of  $2.5 \times 10^5$  cells/ml and were left to recover at 37°C for 30 min. The cells were pelleted and blocked in 40-45 µl normal goat serum, and incubated on ice for 5 min. PE-conjugated antibodies against the extracellular region of TRAIL-R1 and TRAIL-R2 was used to detect TRAIL receptors expressed on the cell surface. 10 µl PE-TRAIL-R1 (Clone: DJR1, eBiosciences) or 5 µl PE-TRAIL-R2 (Clone:DJR2, eBiosciences) was added to the cells to a final volume of 50 µl. 10 µl PE-Mouse IgG1 isotype control (eBiosciences) was used as a negative control. The samples were incubated for 1h on ice in the dark and thereafter washed 3 times in PBS and finally resuspended in 1 ml PBS. The mean fluorescent intensity (MFI) was measured by flow cytometry using an excitation of 488 nm and an emission of 575 nm. The experiment was repeated independently three times.

### ***3.14 Cell surface expression of the TRAIL receptors- Biotin IP***

NCI-H460 cells, cultured in 10 cm dishes were washed twice with ice-cold PBS. In order to biotin label all cell surface proteins, 1 ml biotinylation buffer (50 µg/ml Biotin-7-NHS, 50 mM sodium borate, 150 mM NaCl) was added to the cells and incubated at 4°C for 30 min. The cell culture dish was rocked every 10 min in order to ensure even distribution of biotinylation buffer. The biotinylation reaction was quenched by the addition of 50 µl 500 mM NH<sub>4</sub>Cl to a final concentration of 50 mM for 15 min. The cells were thereafter washed with ice-cold PBS, 1 ml lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, HALT protease/phosphatase inhibitor, 1 mM Vanadate, 1 mM PMSF) was added to the dish and the cells were collected using a cell scraper. The lysates were incubated on ice for 30 min and were centrifuged at 16 000 x g at 4°C for 30 min. A 40 µl aliquot of the cleared lysate was mixed with 20 µl lysis buffer and 60 µl 3x SDS sample buffer and used as input control. The cleared lysate was added to 40 µl streptavidin bead slurry

(beads and lysis buffer, 1:1) in order to immunoprecipitate biotin labeled proteins. The lysate was incubated with the beads o/n at 4°C on a spinning wheel. The beads was washed three times with lysis buffer for 10 min at 4°C on a spinning wheel and resuspended in 40 µl lysis buffer and 70 µl 3x SDS sample buffer. The samples were incubated at 95°C for 3 min, centrifuged at 16,000 x g for 20 s and the supernatant was transferred to a new tube. The input and the IP samples were subjected to SDS-PAGE and blotted for the protein of interest (ie. TRAIL-R1 and TRAIL-R2). Non-biotinylated cells were used as negative control for the immunoprecipitation of unspecific proteins binding to the streptavidin beads.

### ***3.15 DISC formation assay***

The TRAIL DISC complex was isolated by the use of biotinylated TRAIL (see 3.8.4) and streptavidin-sepharose beads. Cells was treated with 30U/ml biotinylated TRAIL for 30 min at 37°C , thereafter collected and lysed for 30 min on ice in Triton lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton-X-100, 1 mM vanadate, complete, protease inhibitor cocktail (Roche) and 1mM NaF). 50 µl aliquots of the lysates were taken as input samples. Lysates were cleared by centrifugation at 16 000 x g for 30 min in a table top centrifuge at 4°C. In order to analyse the total TRAIL receptor levels, 3U/ml biotin-TRAIL was instead added to the cleared lysate, allowing TRAIL to bind all receptors in the cells. One sample without any addition of TRAIL was used as a negative control for the immunoprecipitation. TRAIL-receptors with bound biotinylated TRAIL were precipitated with Streptavidin-Sepharose™ beads (Streptavidin Plus UltraLink Resin, Pierce) over night at 4°C. Beads were washed three times with lysis buffer, resuspended in 60 µl SDS-sample buffer, incubated at 95°C for 3 min and centrifuged at 16,000 x g for 20 s. The supernatant was subject to western blotting (3.6) and blotted for proteins of interest.

### ***3.16 Sucrose gradient for isolation of lipid rafts***

Lipid rafts were isolated by sucrose gradient and ultracentrifugation. One 15 cm cell culture dish with MCF10A cells, at a confluency of approximately 80%,



was used per sample. Cells were treated with 30U/ml TRAIL for 1h. The cells were washed once with ice-cold PBS and collected and lysed in 1 ml lysis buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA and 1% Triton x-100, supplemented with 1 mM vanadate, 1 mM NaF and HALT phosphatase/protease inhibitor). 50 µl aliquots of the lysate were taken as input control. The lysates were incubated on ice for 30 min and homogenized with 10 strokes using a tissue grinder and mixed with 1 ml 85% Sucrose ((w/v) dissolved in lysis buffer without Triton x-100). The lysate and sucrose mixture was transferred to the bottom of a pre-cooled 14 ml open top thin-wall ultracentrifuge tube (cat no: 644060, 14x95mm, Beckman) and carefully overlaid with 7.5 ml 35% sucrose and 3.5 ml 5% sucrose. The samples were centrifuged at 38 000 rpm for 18h at 4°C in a swing out SW40 rotor in an optima L-100XP Beckman centrifuge (acceleration 8, deceleration 8). 1 ml fractions were carefully collected from the top to bottom of the tube and resuspended 1:1 with 3x SDS-sample buffer. Samples were subjected to western blotting as described in 3.6.

### ***3.17 TRAIL receptor clustering assay***

TRAIL receptor clustering was analysed by SDS-PAGE with lysate without reducing agent. One 6 cm cell culture dish was used per sample. Cells were treated with 30U/ml TRAIL for 15min or 1h, washed in PBS and lysed in 0.4 ml cell lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% Glycerol, 1% Triton X-100 supplemented with HALT phosphatase/protease inhibitor, 1mM vanadate and 1 mM NaF). The lysates were incubated on ice for 30 min, thereafter centrifuged at 16 000 x g at 4°C. The cleared lysate was divided in two aliquots and diluted 1:1 with 3x SDS sample buffer with or without 150 mM of the reducing agent DTT. The samples with DTT were incubated at 95°C for 3 min whereas the non-reduced samples were kept on ice. Samples were run on a 7.5% SDS-PAGE gel as described in 3.6.

### ***3.18 Inhibition of clathrin mediated endocytosis***

Clathrin mediated endocytosis was inhibited with 10 µg/ml Chlorpromazine Hydrochloride (CPZ), (Sigma) for 1h prior to treatment with TRAIL for 2h. The

inhibitor was dissolved in DMSO. DMSO alone was added to the non treated samples.

### ***3.19 Inhibition of lysosomal degradation***

H460 cells were treated with the lysosomal inhibitor Chloroquine (Sigma) for 16h at a final concentration of 50  $\mu$ M or 100  $\mu$ M. The cells were thereafter treated with 30U/ml TRAIL for 1.5h.

### ***3.20 Inhibition of kinase activity of EGFR and ErbB2***

MCF10A cells were washed with PBS and DMEM/F12 medium, supplemented with 20 ng/ml EGF, was added. Lapatinib (GlaxoSmithKline), an inhibitor of EGFR and ErbB2 activity, was added at a final concentration of 1  $\mu$ M and incubated for 1 h prior to treatment with TRAIL for 2h.

## **Chapter 4 - Results**

### ***4.1 Ack1 is required for TRAIL induced apoptosis***

#### **4.1.1 Background and aims**

The focus of our research group has mainly been on the tumour suppressor Mitogen inducible gene 6 (Mig6). Mig6 is an adaptor protein that can bind directly to the kinase domain of the ErbB receptors and thereby inhibit the dimerisation and/or phosphorylation of the receptors [149]. Gene targeting of Mig6 in mice have shown that Mig6 is a specific negative regulator of ErbB mediated signalling during development and carcinogenesis [150]. The non-receptor tyrosine kinase, Ack1, has a proline rich, ErbB receptor binding region highly similar to the one of Mig6.

Mig6 has been shown to be a key regulator of apoptosis in mammary epithelial cells (S. Hopkins et al., 2012 *Dev cell*). This raised the question whether Ack1 also may regulate apoptosis. In this work, we have identified Ack1 as a crucial protein for TRAIL induced apoptosis.

#### **The aims were to:**

- a) Generate recombinant TRAIL for the use in subsequent experiments.
- b) Analyse the cell death response in a panel of epithelial cells following TRAIL treatment, in the presence or absence of Ack1 expression.
- c) Investigate if Ack1 regulates apoptosis via other death receptors.
- d) Examine if Ack1 is required for the mitochondrial cell death pathway.
- e) Study whether TRAIL induced survival signalling is increased in Ack1 knockdown cells.
- f) Study the mechanism by which Ack1 regulates TRAIL induced apoptosis

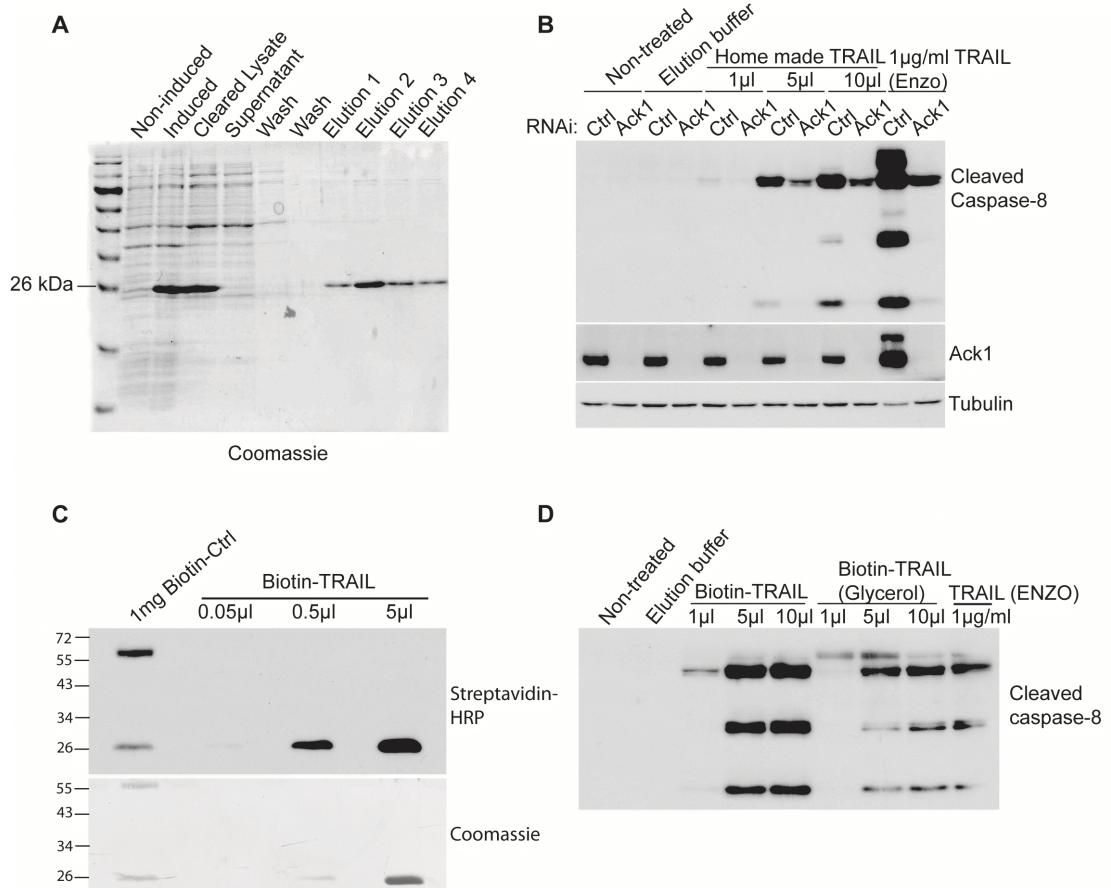
#### **4.1.2 Generation and purification of recombinant TRAIL**

Due to the large quantities of TRAIL required for experiments we sought to generate our own recombinant TRAIL. An expression construct containing the extracellular region of TRAIL (residues 95-281) was expressed in BL21(DE3)RIL *E. Coli* cells. Expression of His-tagged recombinant TRAIL was induced by IPTG and TRAIL was thereafter batch purified with nickel agarose beads and eluted with 100 mM imidazole. The yield and purity was assessed by SDS-PAGE and coomassie brilliant blue staining. Samples from several steps of the purification were run in order to assess the efficiency of the purification. The purity of the protein was satisfactory and elution fractions 1 to 4 were pooled and aliquoted (Figure 9A). In order to control the apoptosis inducing activity of the purified TRAIL, MCF10A cells were treated with three doses of TRAIL for 3h and caspase-8 cleavage was used to assess the apoptotic response. Commercially produced TRAIL (ENZO Life Sciences, cat no. BML-SE722) was used as a positive control at a final concentration of 1 µg/ml. Western blotting results showed that the purified TRAIL was active and induced an adequate amount of cleaved caspase-8, in MCF10A cells, in a dose-dependent manner. The concentration chosen to use in subsequent experiments, defined as 30U/ml, induced approximately one third of the death response compared to commercial TRAIL (100U/ml). Elution buffer alone did not induce caspase-8 cleavage (Figure 9B).

#### **4.1.3 Biotinylation of recombinant TRAIL**

In order to perform DISC co-immunoprecipitation assays (see 3.15) some recombinant TRAIL was labelled with biotin. TRAIL was purified as previously described and conjugated with biotin prior to elution of the protein from the nickel agarose beads. The biotinylation of TRAIL was assessed by western blotting and biotinylated protein was detected using streptavidin conjugated with HRP. As a positive control, 1 mg of a biotin-conjugated antibody was loaded to one of the wells. The purified TRAIL was successfully labelled (Figure 9C). The apoptosis inducing activity was assessed by cleaved caspase-8 levels and was also satisfactory. Two freezing conditions were tested; with or without the addition of 20% glycerol. Results show that storage of biotinylated TRAIL at

-80°C without glycerol conserves the highest death inducing activity (Figure 9D). Freeze thawing of the recombinant TRAIL significantly reduced the activity and was therefore avoided.



**Figure 9. Purification and cell death inducing activity of recombinant TRAIL**

**A.** SDS-PAGE analysis of the purification and expression of recombinant TRAIL. Electrophoresis was performed using a 12.5 % acrylamide gel, and the proteins were stained with coomassie brilliant blue. His-TRAIL protein expression was induced with 1 mM IPTG for 3h at 27°C. The cleared lysate was purified with Ni<sup>2+</sup>-agarose beads and eluted with 100 mM imidazole. **B.** The apoptotic inducing activity of the purified TRAIL was assessed in MCF10A cells by treatment with TRAIL for 3h. Cleavage of caspase-8 was dose dependent. As a positive control, cells were treated with 1 µg/ml commercially made TRAIL (ENZO Life Sciences). Elution buffer alone did not induce cleavage of caspase-8. **C.** TRAIL was labelled with biotin by the addition of Biotin-7-NHS. The biotinylation was assessed by western blot using HRP-conjugated streptavidin. A biotin-conjugated antibody was used as positive control (lane 1). **D.** The cell death inducing activity of the biotin conjugated recombinant TRAIL was assessed by cleavage of caspase-8. Storage of the biotinylated TRAIL at -80°C, without the addition of glycerol, preserves the activity more efficiently compared to addition of 20% glycerol. *Abbreviations: Biotin-7-NHS: D-*

*biotinoyl-ε-aminocaproic acid-N-hydroxysuccinimide ester His: histidine, HRP: horseradish peroxidase, IPTG: Isopropyl-β-D-thio-galactoside, TRAIL: TNF-related apoptosis inducing ligand*

#### **4.1.4 Ack1 knockdown inhibits TRAIL induced apoptosis**

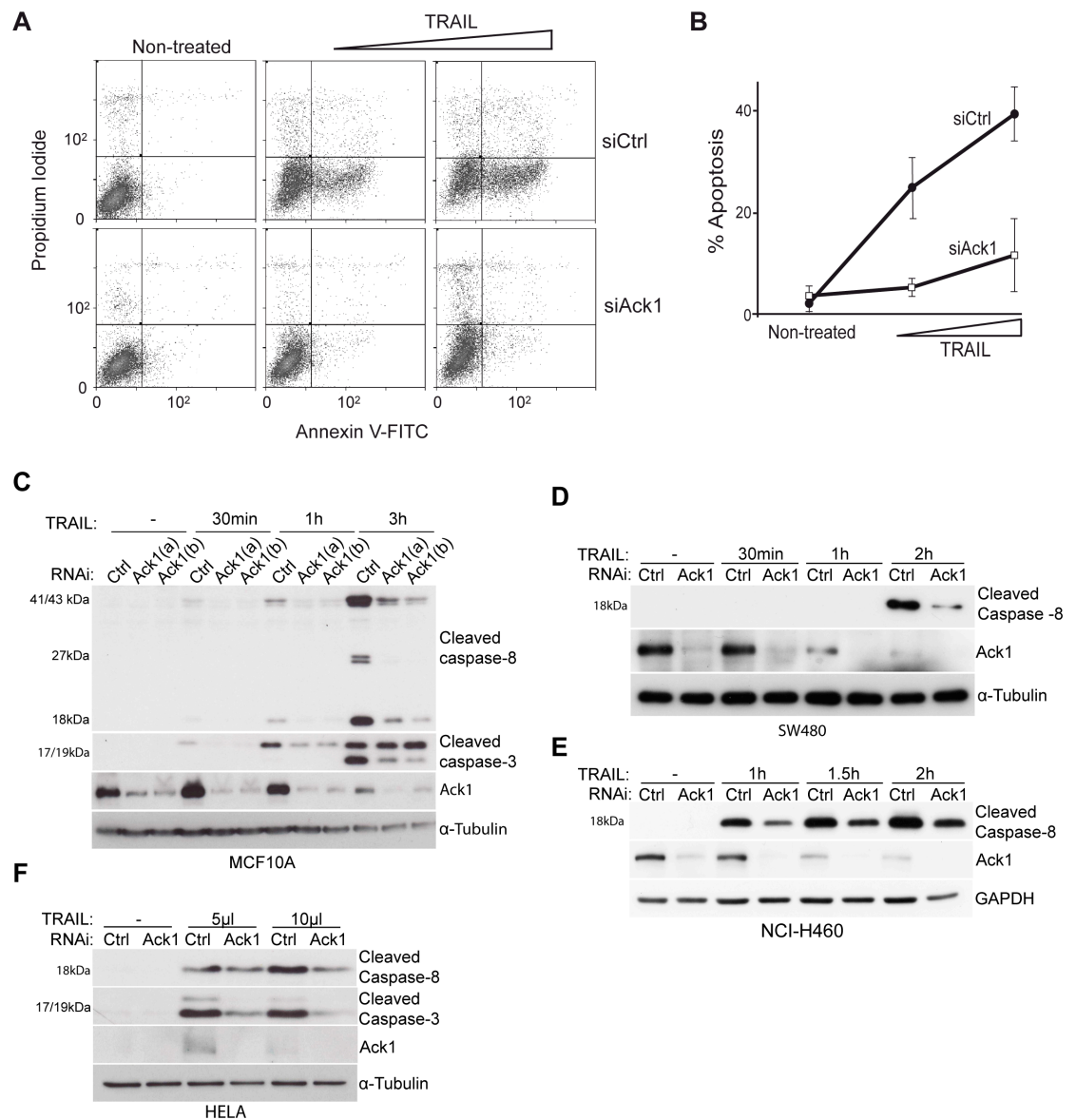
In order to investigate if Ack1 has a role in TRAIL induced apoptosis, the expression of Ack1 was silenced, in human epithelial cells, using siRNA and thereafter treated with TRAIL. The apoptotic response following TRAIL treatment was analysed by measuring the expression of phosphatidylserine (PS) on the cell surface by flow cytometry analysis. Healthy cells express PS in the inner leaflet of the plasma membrane, however during apoptosis; the PS is translocated to the outer membrane of the dying cell. The PS exposure can be detected using a fluorescently labelled phospholipid binding protein, Annexin V, with a high affinity to PS located only on the outer membrane. Annexin V positive cells function as an early marker for apoptosis. Late apoptotic and necrotic cells, which have lost their membrane integrity, are distinguished from early apoptotic cells by propidium iodide (PI) staining.

The TRAIL sensitive mammary epithelial cell line MCF10A was transfected with siRNA targeting Ack1 (siAck1) or non-targeting siRNA (siCtrl). The cells were treated with 2 doses (30U/ml or 60U/ml) of TRAIL for 3h, trypsinised and thereafter stained with FITC-Annexin V and PI. The percentage of early apoptotic (Annexin V positive/PI negative) cells in Ack1 knockdown cells were significantly lower than in control cells with both doses of TRAIL used; 5.4 and 11.5% in Ack1 knockdown cells as compared to 24.8 and 39.4% in control cells ( $p < 0.006$ , students *t*-test). The experiment was repeated three times and a representative dot plot from one of the experiments is shown in Figure 10A. The graph in Figure 10B is an average of the three independent experiments.

To further confirm that Ack1 knockdown inhibits TRAIL induced apoptosis, the levels of cleaved and activated caspase-8 and -3 was analysed by western blotting. Results clearly show that Ack1 knockdown leads to lower levels of both cleaved caspases. Two, non-overlapping, siRNA targeting Ack1 (Ack1a and

Ack1b) were used to confirm the phenotype (Figure 10C). The cells underwent apoptosis in a time and dose dependent manner.

In order to investigate how general this observed phenotype is, the TRAIL treatment experiments were repeated in a panel of human epithelial cell lines, all known to be TRAIL sensitive. Cells were treated with TRAIL for 30 min – 3h, depending on the sensitivity of the cell line, and the apoptotic response was analysed by western blotting. Results show that the epithelial cell lines tested (SW480, HELA and NCI-H460) all had lower levels of cleaved caspase-8 following TRAIL treatment (Figure 10D-F), suggesting that the phenotype is more general for TRAIL-sensitive epithelial cells and not just cell line specific.



**Figure 10. Ack1 is required for TRAIL induced apoptosis in human epithelial cells**

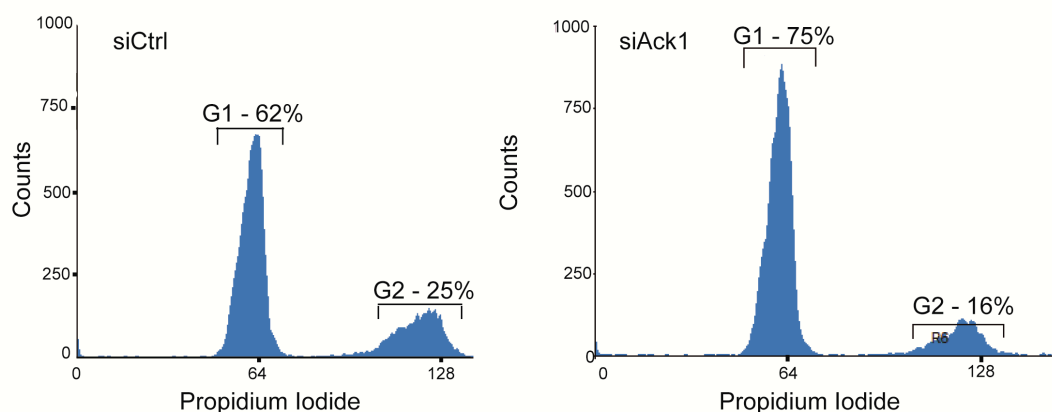
**A.** MCF10A cells transfected with non-targeting (siCtrl) or Ack1 siRNA (siAck1) were either left untreated or treated with 30U/ml or 60U/ml TRAIL for 3h. Cells were stained with FITC-conjugated Annexin V and PI followed by flow cytometry analysis. The percentage of early apoptotic (Annexin V positive/PI negative) cells was significantly lower in Ack1 knockdown cells. Necrotic and late apoptotic cells were positively stained with PI. A representative dot plot from one out of three independent experiments are shown. **B.** An average of three independent Annexin V/PI flow cytometry experiments. Ack1 knockdown cells undergo significantly less apoptosis following TRAIL treatment ( $p > 0.006$ , Students *t*-test). The error bars represent standard deviation. **C.** MCF10A cells were transfected with two non-overlapping siRNA (Ack1a or Ack1b) for the knockdown of Ack1, or transfected with non-targeting siRNA (Ctrl). Cells



were treated with TRAIL for the time points indicated. Western blot analysis show lower levels of cleaved and activated caspase-8 and -3 in Ack1 knockdown cells as compared to control cells. **D-F.** A panel of epithelial cells was treated with 30U/ml TRAIL for the time points indicated. Cleaved caspase-8 levels were lower following Ack1 knockdown in HELA (**D**) SW480 (**E**) and NCI-H460 cells (**F**). *Abbreviations: Ack1: Activated Cdc42-associated kinase 1, FITC: fluorescein isothiocyanate, PI: Propidium Iodide, PS: phosphatidylserine, TRAIL: TNF-related apoptosis-inducing ligand*

#### **4.1.5 Ack1 knockdown leads to a reduction in cell proliferation**

A decreased proliferation rate was observed upon Ack1 knockdown. To further investigate this phenotype, cell cycle profile analysis using propidium iodide (PI) staining and flow cytometry analysis was performed. MCF10A cells were transfected with non-targeting control siRNA (siCtrl) or with Ack1 siRNA (siAck1). The cells were cultured for 48h post-transfection, then trypsinised, fixed in ethanol and stained with PI. The cell cycle profile was analysed by flow cytometry at an excitation of 488 nm and emission of 600 nm. A minimum of 10 000 events/sample was collected and the hypodiploid and diploid DNA peaks were measured. Results show that MCF10A cells, in absence of Ack1, have 13% more cells in G1 as compared to control cells, interpreted as they have a slightly slower proliferation rate (Figure 11). In order to be confident that a difference in confluency would not affect the TRAIL response, 25% more cells were transfected with Ack1 siRNA compared to cells transfected with non-targeting control siRNA to compensate for the lower proliferation rate.

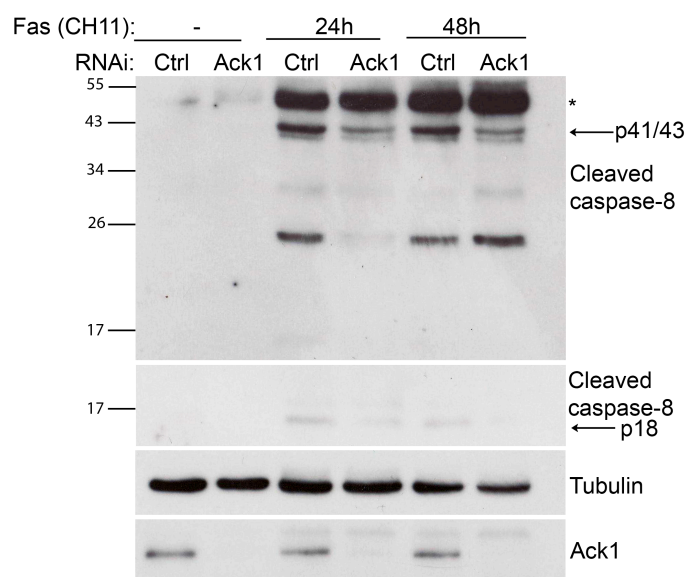


**Figure 11. Decreased proliferation rate in Ack1 knockdown cells**

MCF10A cells were transfected with non-targeting siRNA (siCtrl) or siRNA targeting Ack1 (siAck1) 48h prior to the experiment. Cells were trypsinised, fixed in ethanol and stained with propidium iodide. The cell cycle profile was analyzed by flow cytometry. Ack1 knockdown resulted in a 13% increase of cells in G1 as compared to control transfected cells. *Abbreviations: Ack1: Activated Cdc42-associated kinase 1, PI: propidium iodide*

#### 4.1.6 Regulation of Fas-mediated apoptosis by Ack1

To elucidate the specificity of Ack1 on regulating apoptosis via the death receptor pathway, we wanted to investigate if Ack1 knockdown also inhibits Fas-induced apoptosis. MCF10A cells were treated with 2  $\mu$ g/ml Fas receptor-activating antibody for 24 to 48h. Cell death was assessed by western blotting of cleaved caspase-8. Results show that there was a slight decrease in the levels of cleaved caspase-8 in Ack1 knockdown cells, suggesting that Ack1 might play a role in the regulation of Fas induced apoptosis. The ~50 kDa band detected in the cleaved caspase-8 blot is most likely the heavy chain of IgG from the antibody used for the treatment (Figure 12).

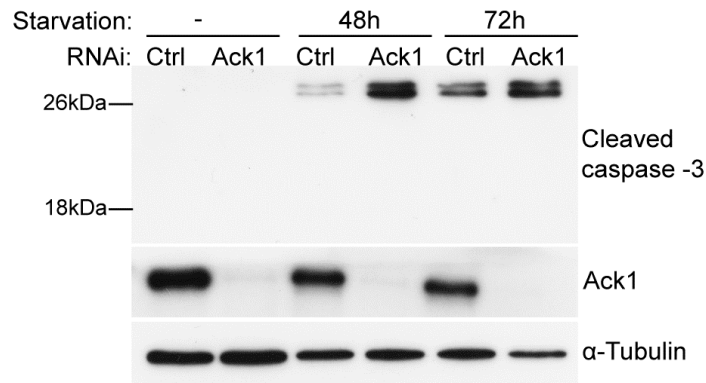


**Figure 12. Ack1 regulates Fas-induced apoptosis**

MCF10A cells were transfected with non-targeting (Ctrl) or Ack1 siRNA and cultured for 48h. The cells were treated with 2 µg/ml Fas-activating antibody for 24 to 48h and the cell death response was analysed by western blotting of cleaved caspase-8 levels. A slight decrease in cleaved caspase-8 was seen in Ack1 knockdown cells as compared to control transfected cells. \* The ~50 kDa band detected in the cleaved caspase-8 blot is most likely IgG from the antibody used for the treatment. *Abbreviations: Ack1: Activated Cdc42-associated kinase 1*

#### 4.1.7 Ack1 is not required for apoptosis via the mitochondrial pathway

In order to investigate if Ack1 only regulate the death receptor pathway or if it also plays a role in the mitochondrial pathway, experiments activating the mitochondrial pathway were performed. Serum starvation and growth factor deprivation is thought to induce cell death via the mitochondrial pathway. MCF10A cells were cultured without serum and growth factors for 48h to 72h and apoptosis was assessed by cleaved caspase-3 levels using western blotting. The cleaved caspase-3 levels were equal or even elevated in the Ack1 knockdown cells compared to control transfected cells (Figure 13). These results suggest that Ack1 promotes apoptosis specifically via the death receptor pathway. The increase of caspase-3 cleavage seen in Ack1 knockdown cells was not further investigated.



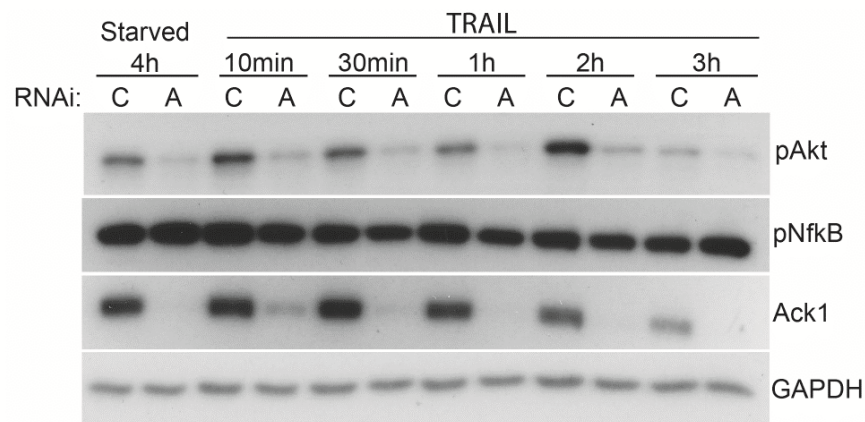
**Figure 13. Ack1 is not required for apoptosis via the mitochondrial pathway**

Ack1 expression was silenced with siRNA and non-targeting siRNA was used as control. The experiment was performed 48h post-transfection. MCF10A cells were cultured devoid of serum and growth factors for 48 to 72h. Apoptosis was assessed by western blotting of cleaved caspase-3 levels. Ack1 knockdown does not inhibit the apoptotic response via the mitochondrial pathway. *Abbreviations: Ack1: Activated Cdc42-associated kinase 1*

#### **4.1.8 Ack1 knockdown does not promote survival signalling pathways via the TRAIL receptors**

Following TRAIL ligand binding, alternative signalling pathways promoting anti-apoptotic activities, such as migration, invasion and inflammation can be activated, via the formation of a second complex following the assembly of the DISC. Two proteins known to be activated are NfκB and Akt [151, 152]. In order to investigate if Ack1 knockdown leads to a shift from death inducing signalling towards survival signalling, MCF10A cells were serum starved for 4h and thereafter treated with TRAIL for 3h. The levels of phosphorylated NfκB (pNfκB) and Akt (pAkt) was analysed by western blotting. Results show that there is no increase in phosphorylation of NfκB or Akt in response to TRAIL in MCF10A cells. In addition, there was no difference in the pNfκB levels in Ack1 knockdown cells as compared to control transfected cells (Figure 14). In contrast, Ack1 knockdown leads to significantly lower levels of pAkt both in non-treated and TRAIL treated cells. Ack1 is known to phosphorylate Akt [116], so the decrease was therefore expected and independent of TRAIL (Figure 14). These results suggest that Ack1 knockdown does not lead to an increase in

survival signalling via NfκB and Akt signalling pathways in response to TRAIL treatment.



**Figure 14. Ack1 knockdown does not lead to increased TRAIL induced NfκB and Akt activation**

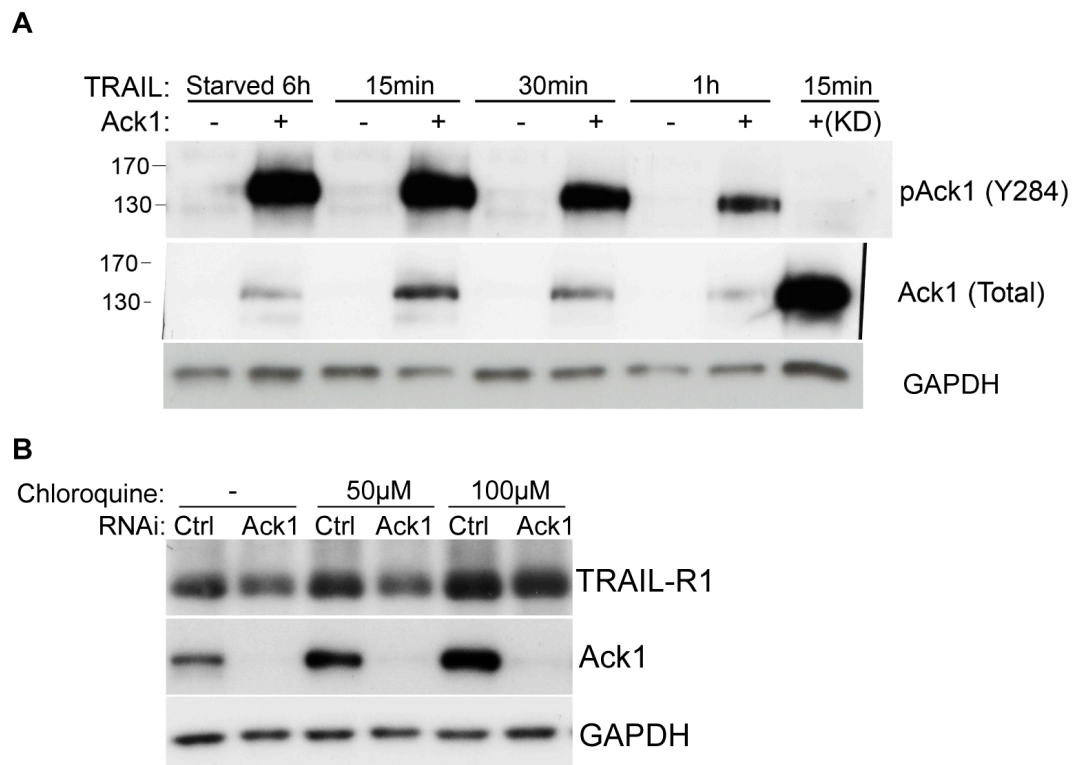
MCF10A cells were transfected with control siRNA (C) or siRNA targeting Ack1 (A). Experiments were performed 48 h post transfection. Cells were serum starved for 4h and thereafter treated with 30U/ml TRAIL for 3h. Western blot analysis shows no increased phosphorylation of NfκB in the absence of Ack1. The phosphorylation levels of Akt were lower in Ack1 knockdown cells, independently of TRAIL treatment. *Abbreviations: Ack1: Activated Cdc42-associated kinase 1, NfκB: Nuclear factor-κB, p: phosphorylation, TRAIL: TNF-related apoptosis-inducing ligand*

#### 4.1.9 Ack1 is dephosphorylated in response to TRAIL

In order to investigate the activity of Ack1 in response to TRAIL, wild type or kinase dead (KD) Ack1 was ectopically expressed in H460 cells and thereafter serum starved for 6h and treated with TRAIL for the time points indicated. Significant levels of phosphorylated Ack1 were still detected in serum starved cells. Interestingly, TRAIL treatment reduced the phosphorylation levels in a time dependent manner, suggesting that Ack1 is dephosphorylated in response to TRAIL. Phosphorylation of kinase dead Ack1 was not detected, confirming the specificity of the pAck1 antibody (Figure 15A).

A decrease of the total levels of Ack1 following TRAIL treatment have been detected in many experiments performed (for example in Figure 10 and Figure 18). In addition, knockdown of Ack1 in some cases leads to a decreased level of TRAIL-R1, it is unclear why this difference is only detected in some

experiments and not in others. One possible explanation is that Ack1 transiently regulates TRAIL-R1 stability and/or trafficking. In order to investigate the stability and degradation of Ack1 and TRAIL-R1, lysosomal degradation was inhibited using the inhibitor Chloroquine at a concentration of 50  $\mu$ M to 100  $\mu$ M for 16h. Ack1 and TRAIL-R1 levels were analysed by western blotting. Results show that lysosomal inhibition increased both the TRAIL-R1 and Ack1 levels, suggesting that both proteins are degraded via a lysosome dependent pathway. Non-treated Ack1 knockdown cells had slightly lower levels of TRAIL-R1 compared to control transfected cells, which have been observed in other experiments performed. The difference in protein levels are however restored by the higher dose of Chloroquine (Figure 15B).

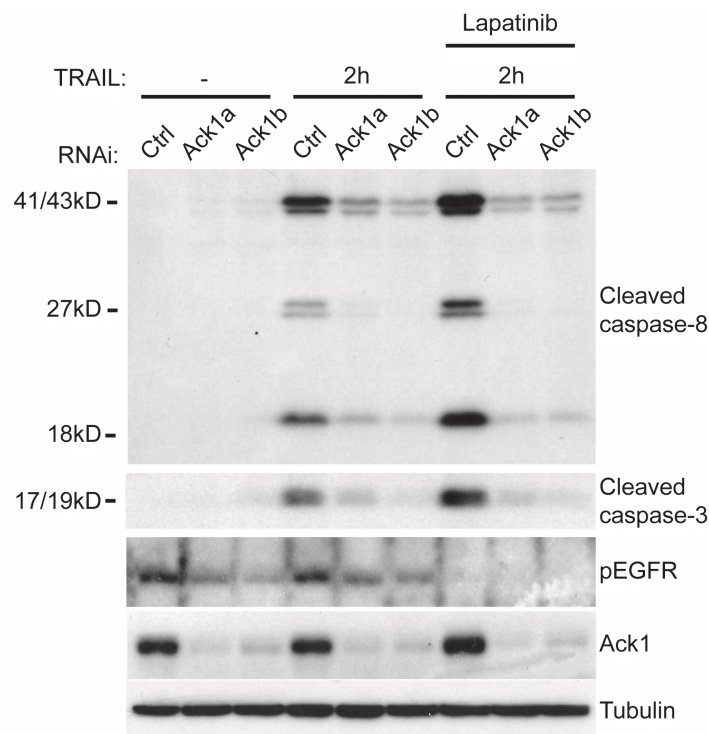


**Figure 15. Ack1 is dephosphorylated in response to TRAIL.**

**A.** H460 cells were either transfected with an empty vector (-), with an Ack1 wild type (+) or an Ack1 kinase dead (+KD) expression vector. The cells were serum starved for 6h and treated with TRAIL for the time points indicated. **B.** Lysosomal degradation was inhibited with 50  $\mu$ M or 100  $\mu$ M Chloroquine for 16h, where indicated. Protein levels of TRAIL-R1 and Ack1 are increased after lysosome inhibition with Chloroquine.

#### 4.1.10 Ack1 promotes TRAIL induced apoptosis independently of ErbB signalling

Ack1 has previously been described to interact with and regulate EGFR signalling, we therefore wanted to investigate if Ack1 controls TRAIL induced apoptosis via an ErbB signalling dependent mechanism. This was addressed by treating the cells with the epidermal growth factor receptor (EGFR) and ErbB2 inhibitor Lapatinib followed by TRAIL treatment for 2h. Lapatinib inhibited the phosphorylation of EGFR effectively, visualised by the loss of phospho-EGFR in Lapatinib treated cells analysed by western blotting (Figure 16 lane 7-9). Inhibition of ErbB signalling failed to restore the levels of cleaved caspase-8 and -3 in Ack1 knockdown cells following TRAIL treatment suggesting that Ack1 regulates TRAIL induced apoptosis independently of ErbB signalling (Figure 16).



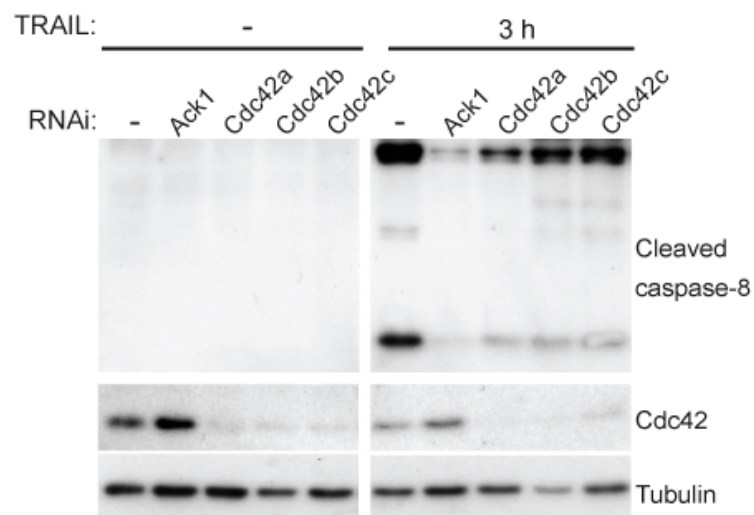
**Figure 16. Ack1 promotes TRAIL induced apoptosis independently of EGFR and ErbB2 activity.**

Ack1 expression was silenced in MCF10A cells by two independent siRNA (Ack1a and Ack1b), non-targeting siRNA was used as control. The kinase activity of EGFR and ErbB2 was inhibited with 1  $\mu$ M Lapatinib, where indicated, and thereafter treated with TRAIL for 2h. Inhibition of ErbB signalling did not restore the cleaved caspase-8 and -3 levels in Ack1 knockdown cells.

#### ***4.1.11 Cdc42 is required for TRAIL induced apoptosis***

Ack1 is a downstream effector of Cdc42. Striking morphological changes was seen in HELA cells when Ack1 was silenced, although not in other cell lines examined (data not shown). The cells were elongated and resembled the phenotype described for Cdc42 knockdown cells. We therefore wanted to investigate if the Ack1 function in TRAIL induced apoptosis is Cdc42 dependent.

MCF10A cells were transfected with siRNA targeting Ack1, Cdc42 or with a non-targeting control. The Cdc42 expression was silenced with three independent non-overlapping siRNA (Cdc42 a, b, c). The cells were treated with TRAIL for 3h and samples were subjected to western blotting. Results show that cleaved caspase-8 levels were lower in both Cdc42 and Ack1 knockdown cells compared to control cells (Figure 17). This data suggest that Cdc42 also is required for TRAIL induced apoptosis, either directly or indirectly by regulating/activating Ack1.



**Figure 17. Cdc42 knockdown inhibits TRAIL induced apoptosis**

MCF10A cells were transfected with non-targeting control, Ack1 and Cdc42 siRNA. Expression of Cdc42 was knocked down with three non-overlapping siRNA. The cells were thereafter treated with 30U/ml TRAIL for 3h. Lower levels of caspase-8 were detected in both Ack1 and Cdc42 knockdown cells compared with cells transfected with non-targeting siRNA.



*Abbreviations: Ack1: Activated Cdc42-associated kinase 1, Cdc42: Cell division cycle 42, GTP binding protein, TRAIL: TNF-related apoptosis-inducing ligand*

#### **4.1.12 Discussion**

In this work, a novel role of Ack1 in death receptor signalling has been discovered; demonstrating that the non-receptor tyrosine kinase, Ack1, is required for TRAIL induced apoptosis in human epithelial cells.

Recombinant TRAIL was generated in order to perform several assays requiring large quantities of TRAIL and the death-inducing activity determined empirically. Ack1 expression was silenced by transient siRNA transfection and more than ~80% knockdown of Ack1 was achieved, confirmed by western blotting. This degree of knockdown was necessary to prevent TRAIL induced apoptosis (data not shown). The levels of Ack1 varied between cell lines and hence the amount of siRNA that needed to be used: 12 mM for most of the cell lines (MCF10A, HELA and SW480) but 30 nM for the high expressing NCI-H460 cell line.

A decreased proliferation rate was observed upon Ack1 knockdown. Cell cycle analysis by PI staining of genomic DNA and flow cytometry confirmed that the Ack1 knockdown population had more cells in G1 (75%) compared to control transfected cells (62%) (Figure 11). The effect on cell proliferation following Ack1 knockdown has been described previously and it has been suggested that Ack1 promotes proliferation by activating mitogenic signalling pathways such as the Akt signalling cascade [153]. Due to the decreased proliferation rate following Ack1 knockdown, 25% more cells were transfected with the Ack1 siRNA oligo in order to ensure that the cell confluency at the time of TRAIL treatment was similar in control and knockdown cells.

Apoptosis via the TRAIL signalling pathway was induced in a time and dose dependent manner using the recombinant TRAIL produced. The apoptotic response was assessed by Annexin V/PI staining and flow cytometry analysis.

The results showed that Ack1 knockdown leads to a significant decrease in early apoptotic (Annexin V positive/PI negative) cells following TRAIL treatment in the mammary epithelial cell line MCF10A (Figure 10A-B). The cell death response was also analysed with western blotting of cleaved and activated caspase-8 and caspase-3, considered to be key caspases in death receptor signalling. Ack1 was silenced in MCF10A cells using two independent non-overlapping siRNA and treated with TRAIL for 3h. Results show that Ack1 knockdown leads to significantly lower levels of both cleaved caspase-8 and caspase-3 (Figure 10C). The phenotype was confirmed in an additional three human epithelial cell lines (SW480, HELA and NCI-H460), suggesting that the role of Ack1 in TRAIL induced apoptosis is more general for TRAIL-sensitive epithelial cells (Figure 10D-F). The cell lines used in this work were mainly the MCF10A cell line and the human lung carcinoma cell line, NCI-H460. Both cell lines are sensitive to TRAIL and NCI-H460 express relatively high levels of Ack1 and TRAIL-R1 compared to MCF10A cells, assessed by western blotting (data not shown).

A direct role of Ack1 in apoptosis has not previously been reported. However in contrast to our results, Ack1 has been described to promote cell survival of cancer cells. Ack1 was identified as a survival kinase in a large-scale RNAi screen targeting all known kinases [130]. Apoptosis was induced by DNA damaging drugs, activating the mitochondrial pathway. When Ack1 is knocked down 2.5 fold more cell death is observed compared to control transfected cells. However the underlying molecular mechanism and the phenotype was not characterised further. These results agree with the starvation experiment performed here where cleaved caspase-3 levels were slightly elevated in Ack1 knockdown cells demonstrating that Ack1 is not required for apoptosis via the mitochondrial pathway, instead rather might prevent cell death (Figure 13).

To address if Ack1 specifically regulates TRAIL induced apoptosis or whether it also can regulate other death receptor pathways, such as FasL induced apoptosis, MCF10A cells were treated with a Fas-activating antibody. The antibody induced apoptosis in MCF10A cells, although not as efficiently as with

TRAIL treatment. There was a slight decrease of cleaved caspase-8 in Ack1 knockdown cells after 24h of TRAIL treatment, suggesting that Ack1 may also regulate Fas induced apoptosis to some extent (Figure 12). The effect was however more striking with TRAIL treatment and that pathway will be further studied in this work.

TRAIL is able to activate anti-apoptotic signalling pathways promoting cellular events such as proliferation, migration and inflammation in some circumstances. Two proteins known to be activated by TRAIL are Akt and NfκB. Western blot analysis of phosphorylated forms of Akt and NfκB were performed in order to investigate if Ack1 knockdown might lead to a diversion of the TRAIL receptor signalling, from death inducing, to an anti-apoptotic signalling mode. MCF10A cells were starved for 4h, treated with TRAIL and subjected to western blotting. TRAIL did not activate either Akt or NfκB in the MCF10A cells, since the phosphorylation levels in TRAIL treated cells were comparable to serum starved cells. Moreover, Ack1 knockdown did not influence the phosphorylation status of NfκB. pAkt levels were significantly decreased in Ack1 knockdown cells, independent of TRAIL treatment (Figure 14). This may be consistent with a previous report showing that Akt is a direct phosphorylation substrate of Ack1. Ack1 phosphorylates Akt at Tyr176 resulting in plasma membrane translocation of Akt and promotes Thr308/Ser473-phosphorylation leading to Akt activation [116].

Overexpressed Ack1 was still to a large extent phosphorylated even after serum starvation for 6h. It has previously been reported that one pool of Ack1 is constitutively activated in the cell even in the absence of stimuli [110] alternatively that the overexpression itself caused auto activation of the protein. Although, the phosphorylation of Ack1 decreased after TRAIL treatment in a time dependent manner (Figure 15A), it is still unclear if it mainly depends on a reduction in total Ack1 levels or a specific dephosphorylation of the protein. It could suggest that Ack1 needs to be inactive in order to perform its regulatory role in TRAIL induced apoptosis. To further investigate the reduction in protein levels of both TRAIL-R1 after Ack1

knockdown and reduction in Ack1 in TRAIL treated cells, cells were treated with the lysosomal inhibitor, Chloroquine. Both TRAIL-R1 and Ack1 levels were increased after Chloroquine treatment (Figure 15B). These results confirm another study claiming that Ack1 is degraded via the lysosome [129]. Others have although reported that Ack1 are degraded by the proteasome [128].

Ack1 interacts with the EGFR and regulates ErbB signalling, potentially by inducing degradation of EGFR or preserving EGFR on the cell surface, however the mechanism is not yet fully understood [119-121]. We therefore wanted to investigate whether the role of Ack1 is dependent on ErbB signalling. The inhibitor Lapatinib was used to inhibit the kinase activity of EGFR and ErbB2 followed by TRAIL treatment for 2h. Results show that Ack1 regulates TRAIL induced apoptosis independently of EGFR/ErbB2 activity since treatment with the inhibitors Lapatinib did not restore the levels of cleaved caspases in Ack1 knockdown cells (Figure 16). Ack1 is also a known downstream effector of Cdc42 and experiments were therefore performed in order to investigate if the role of Ack1 in TRAIL induced apoptosis is Cdc42 dependent. Indeed, knockdown of Cdc42 resulted in lower levels of cleaved caspase-8 following TRAIL treatment, suggesting that Cdc42 also is important for TRAIL induced apoptosis (Figure 17). The Cdc42 expression was silenced with three independent, non-overlapping, siRNA, in order to rule out possible off-target effects.

Ideally, to verify the target specificity of the Ack1 siRNAs a rescue experiment should be performed and was attempted. By ectopic expression of siRNA resistant Ack1 in an Ack1 knockdown background the cell death response should be restored. Several point mutants of Ack1 have been generated by site-directed mutagenesis. The mutations either impair the tyrosine kinase activity or disrupt protein-protein interaction domains, such as Cdc42 and clathrin binding (3.5.3). The point mutants would serve as a useful tool to determine if kinase activity or interactions with other proteins, such as Cdc42, is required. Unfortunately, due to technical difficulties, ectopic expression of the mutants has not been successful. Transient transfection with both siRNA and plasmid

DNA were either cytotoxic or an inadequate amount of overexpression was obtained. Another approach was to generate stable cell lines expressing siRNA resistant Ack1, however this also failed, presumably due to selection against Ack1 expressing cells.

## ***4.2 Trafficking and sub-cellular localisation of the TRAIL receptors***

### **4.2.1 Background and aims**

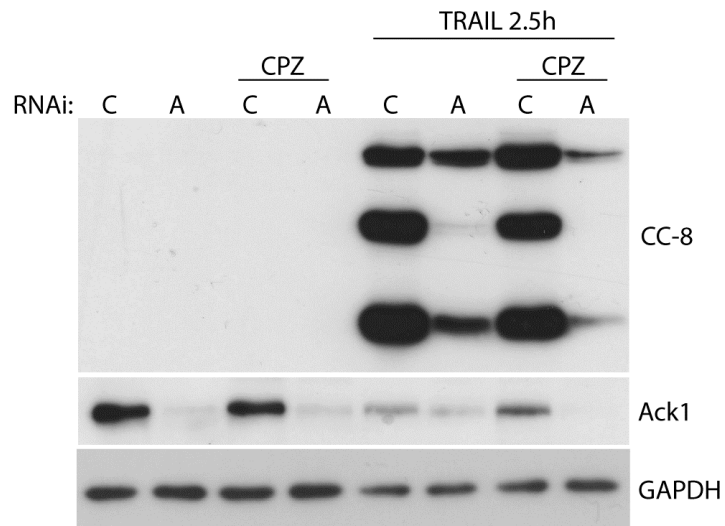
Trafficking and sub-cellular localisation of the TRAIL receptors have been shown to be important for the cell death response. For example, loss of cell surface expression of the TRAIL receptors due to constitutive endocytosis is a known mechanism of TRAIL resistance [74]. Ack1 is suggested to regulate the trafficking and endocytosis of ErbB receptors and Ack1 interacts with the heavy chain of clathrin and the adaptor protein AP-2 [124]. However the mechanism by which Ack1 regulates endocytosis and trafficking is not fully understood. A potential role for Ack1 in the trafficking of the TRAIL receptors has been investigated. In addition, Ack1 was found to be crucial for cell-cell junction stability, although this did not seem to be directly linked to the role of Ack1 in TRAIL induced death response.

#### **The aims were to:**

- a) Examine the putative role of clathrin mediated endocytosis in TRAIL induced apoptosis and a potential regulatory role of Ack1 in endocytosis.
- b) Investigate if Ack1 regulates the cell surface expression of the TRAIL receptors by studying subcellular localisation of the TRAIL receptors in the absence and presence of Ack1.
- c) Elucidate whether cell-cell junctions are important for TRAIL receptor dynamics and cell death response.

#### 4.2.2 Inhibition of clathrin mediated endocytosis does not affect the TRAIL cell death response

In order to elucidate if Ack1 control the TRAIL response by regulating the endocytosis of the TRAIL receptors, clathrin mediated endocytosis was inhibited with the established inhibitor Chlorpromazine (CPZ) followed by treatment with TRAIL for 2.5h. Western blotting results show that the cleaved caspase-8 levels were equal in the cells treated with CPZ compared to cells treated only with TRAIL. In addition, the reduction of caspase-8 cleavage in Ack1 knockdown cells were not restored in CPZ treated cells, suggesting that Ack1 regulates TRAIL induced apoptosis independently of clathrin mediated endocytosis (Figure 18).



**Figure 18. Ack1 regulates TRAIL induced apoptosis independently of clathrin mediated endocytosis.**

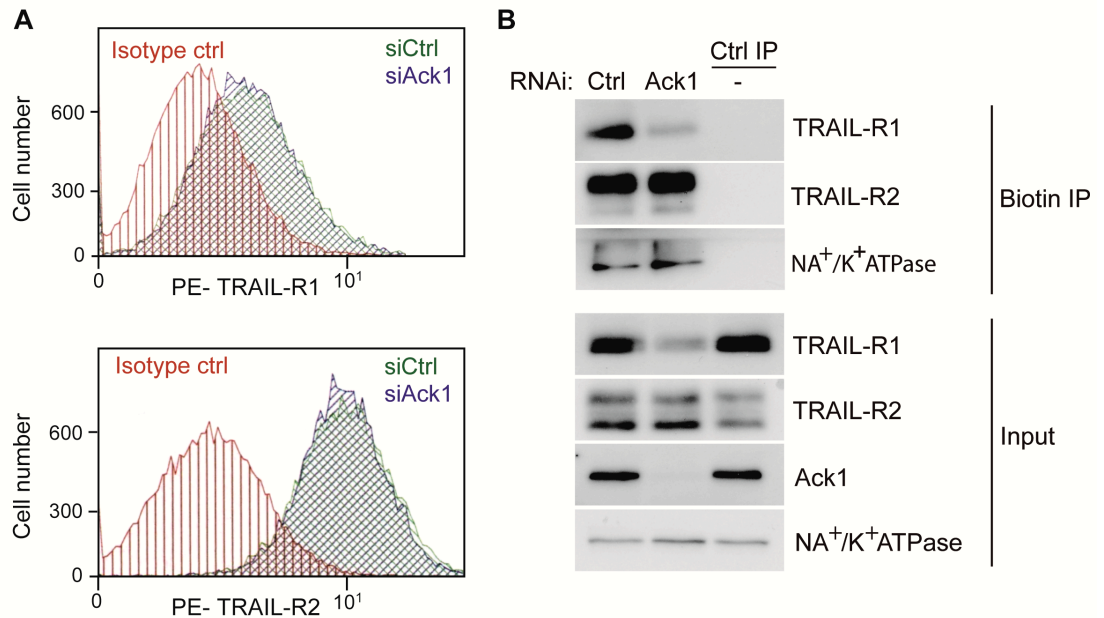
MCF10A cells transfected with control siRNA or siRNA targeting Ack1, were treated with 10  $\mu$ g/ml Chlorpromazine for 1h where indicated, followed by TRAIL treatment for 2.5h. There was no difference in cell death response, assessed by the cleavage of caspase-8, in cells treated with Chlorpromazine, in either control or Ack1 knockdown cells, compared to cell treated with TRAIL alone. *Abbreviations: CPZ: Chlorpromazine, Ack1: Activated Cdc42-associated kinase, TRAIL: TNF-related apoptosis-inducing ligand*

#### **4.2.3 Ack1 knockdown does not alter the cell surface expression of the TRAIL receptors**

The cell surface expression of TRAIL-R1 and -R2 was compared between control and Ack1 knockdown cells to elucidate whether Ack1 is required for the membrane localisation of the receptors. MCF10A cells, transfected with control or Ack1 siRNA, were trypsinised and blocked in goat serum. The cells were thereafter incubated with phycoerythrin (PE) conjugated antibodies targeting the extracellular domain of TRAIL-R1, -R2 or incubated with a PE-IgG1 isotype control for 1h. Cell surface expression of the TRAIL receptors was analysed by flow cytometry using excitation and emission wavelengths of 488 and 575 nm respectively. The mean-fluorescence intensity (MFI) value was analysed using the Summit 4.3 software and the PE-intensity from the TRAIL receptor antibodies binding cell surface receptors was compared to the intensity of the isotype negative control. The MCF10A cells express both TRAIL receptors on the cell surface (Figure 19). Furthermore, no difference in cell surface expression following Ack1 knockdown was detected, since the PE-intensities were comparable. The data presented is a representative graph from three independent experiments. Successful knockdown was confirmed by western blotting (data not shown).

To further confirm that the cell surface expression is not effected in Ack1 knockdown cells a Biotin-IP of cell surface protein was performed. Cell surface proteins were labelled with biotin-7-NHS, binding free amine groups. The biotinylated proteins were thereafter immunoprecipitated with streptavidin beads, subjected to western blotting and probed for the protein of interest (ie. TRAIL-R1 and -R2). Input samples with total protein content were run as a control. Comparable levels of biotinylated TRAIL-R2 were immunoprecipitated in control and Ack1 knockdown cells, confirming that the TRAIL-R2 cell surface expression is equal in Ack1 knockdown cells compared to control cells. The total levels of TRAIL-R1 varied between the control and Ack1 knockdown cells so it is therefore not feasible to draw conclusion regarding the cell surface

expression of TRAIL-R1 in these experiments. Na<sup>+</sup>/K<sup>+</sup> ATPase was used as a loading control (Figure 19C).



**Figure 19. Ack1 knockdown does not alter the cell surface expression of the TRAIL receptors**

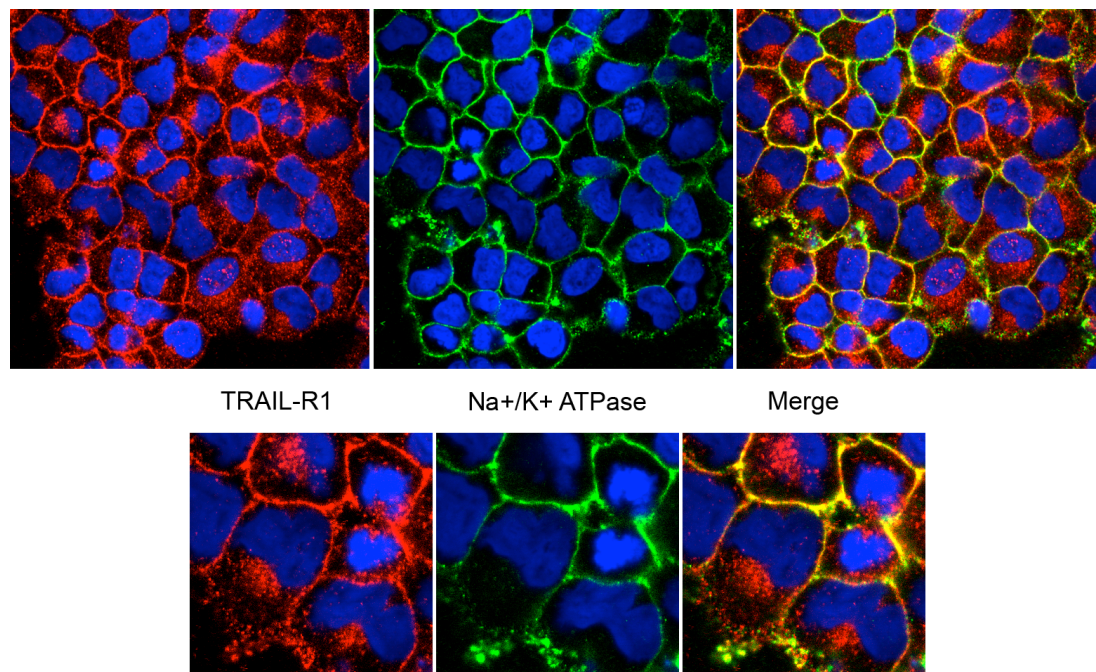
**A.** Flow cytometry analysis of cell surface expression of TRAIL-R1 and TRAIL-R2 in MCF10A cells. Cells were incubated with PE-conjugated antibodies targeting the ligand-binding domain of TRAIL-R1 and R2 or with an IgG isotype control. There was no difference in the cell surface expression in Ack1 knockdown cells compared to control transfected cells. Na<sup>+</sup>/K<sup>+</sup> ATPase was used as a loading control. **B.** Cell surface proteins were labelled with biotin and precipitated with streptavidin beads. Total cell lysates were used as input control. Samples were subjected to SDS PAGE and Western blot and probed for TRAIL-R1 and R2. *Abbreviations: Ack1: Activated Cdc42-associated kinase, Biotin-7-NHS: D-biotinoyl-ε-aminocaproic acid-N-hydroxysuccinimide ester, PE: phycoerythrin, TRAIL-R: TNF-related apoptosis-inducing ligand receptor*

#### 4.2.4 TRAIL-R1 is localised at cell-cell contacts

In addition to previous experiments presented in 4.2.3, the cell surface expression of the TRAIL receptors was also investigated by immunocytochemistry (ICH). NCI-H460 cells were plated on collagen coated glass coverslips and thereafter stained for TRAIL-R1 and the sodium/potassium adenosine triphosphatase (Na<sup>+</sup>/K<sup>+</sup> ATPase), commonly used as a plasma membrane marker, localised mainly on the basolateral surface of



most epithelial cells. Confocal imaging of the cells show the Na<sup>+</sup>/K<sup>+</sup> ATPase primarily located at the plasma membrane at cell-cell contacts with limited staining where cell-cell contacts are absent. TRAIL-R1 share highly similar staining pattern and co-localise with the Na<sup>+</sup>/K<sup>+</sup> ATPase at cell-cell borders (Figure 20). Single plane confocal images were acquired at 63x magnification.



**Figure 20. TRAIL-R1 is mainly localised at cell-cell contacts**

Single plane confocal images of NCI-H460 cells stained with TRAIL-R1 (red) and the membrane marker Na<sup>+</sup>/K<sup>+</sup> ATPase (green) at 63x magnification. TRAIL-R1 and Na<sup>+</sup>/K<sup>+</sup> ATPase co-localise at cell-cell contacts.

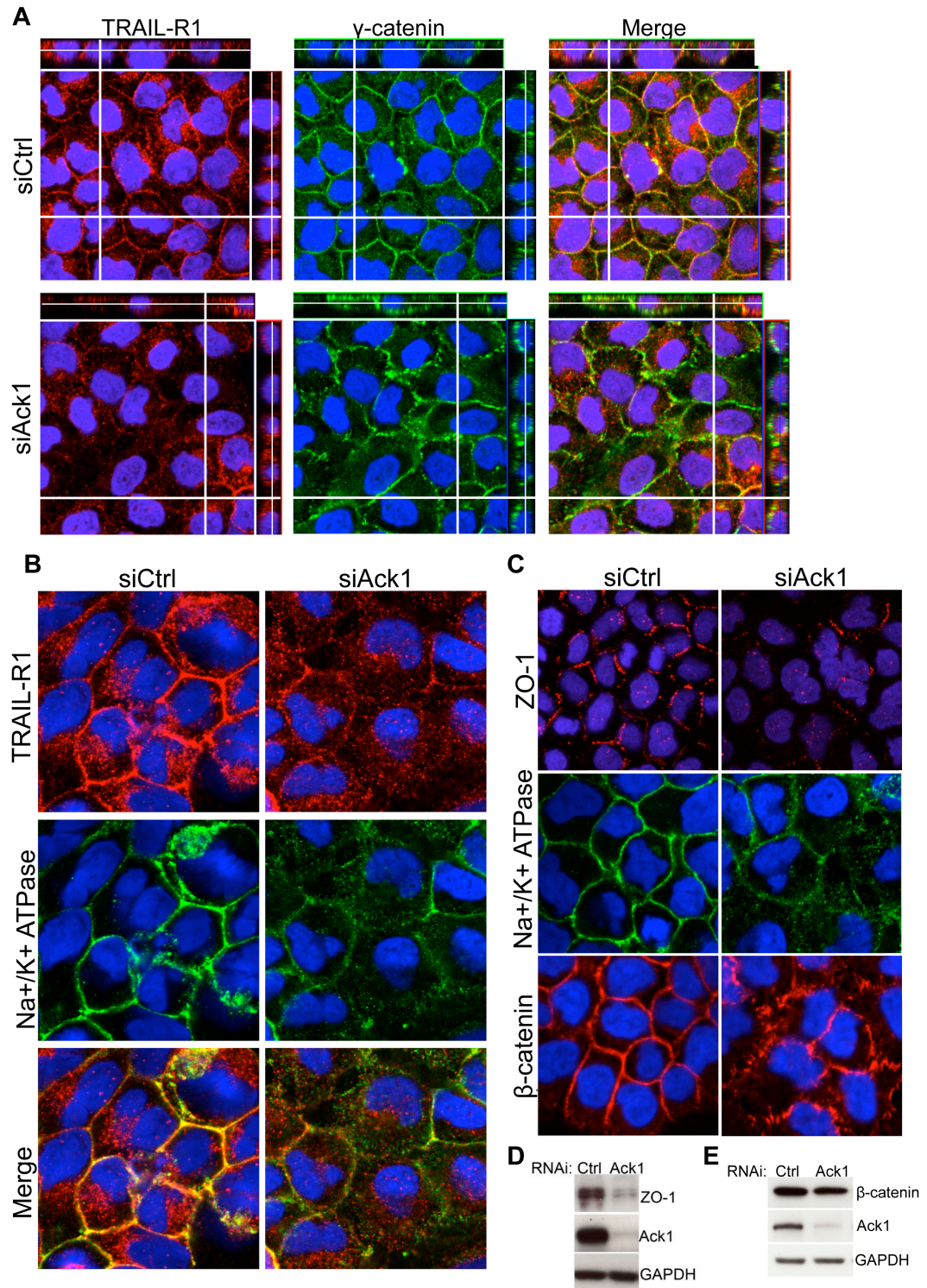
#### **4.2.5 Ack1 knockdown leads to impaired cell-cell junction formation/stability and mislocalisation of TRAIL-R1**

In order to investigate whether the sub-cellular localisation of TRAIL-R1 were altered in the absence of Ack1, ICH experiments were repeated with Ack1 knockdown cells. The adherens/desmosomal protein  $\gamma$ -catenin was used as a cell-cell junction marker. Interestingly, it was observed that the Ack1 knockdown cells had a disrupted sub cellular localisation of both TRAIL-R1 and  $\gamma$ -catenin (Figure 21). The expression of the proteins on the cell surface was to a large extent lost and the proteins were more internalised and present in a

disorganised pattern. By analysing the confocal images in an orthogonal view it was possible to see that both  $\gamma$ -catenin and TRAIL-R1 were localised more at the apical side of the cells after Ack1 knockdown. Moreover, the sub-cellular localisation of  $\text{Na}^+/\text{K}^+$  ATPase was also mislocalised in a similar manner to  $\gamma$ -catenin (Figure 21B). The apical localisation of junctional protein observed could suggest that epithelial cell polarity is disrupted in Ack1 knockdown cells.

Knockdown of Ack1 in NCI-H460 cells also resulted in a significant mislocalisation of  $\beta$ -catenin and ZO-1, an important adaptor protein mainly in tight junctions.  $\beta$ -catenin was significantly mislocalised and showed a staining pattern similarly to the staining pattern of  $\gamma$ -catenin (Figure 21C). The total protein levels of  $\beta$ -catenin did not change, confirmed by western blotting (Figure 21E). In contrast, knockdown of Ack1 leads to a downregulation of ZO-1 protein levels (Figure 21C-D)

These observations lead to further investigations of a possible link between disrupted cell-cell junction and decreased sensitivity of TRAIL induced apoptosis in Ack1 knockdown cells.



**Figure 21. Ack1 knockdown cells lead to mislocalisation of TRAIL-R1 and impaired cell-cell junction stability/formation**

**A.** Single plane confocal images of NCI-H460 cells stained with TRAIL-R1 (red) and  $\gamma$ -catenin (green) at 63x magnification. Orthogonal view shows apical staining of the receptor in Ack1 knockdown cells, which is not seen in control cells. **B.** Stainings of TRAIL-R1 (red) and the

membrane marker Na<sup>+</sup>/K<sup>+</sup> ATPase (green). **C.** Cell-cell junction proteins are mislocalised in Ack1 knockdown cells. Single plane confocal images of NCI-H460 cells stained with ZO-1 (red), Na<sup>+</sup>/K<sup>+</sup> ATPase (green) and  $\beta$ -catenin (red). **D.** Western blotting of ZO-1 levels. ZO-1 is downregulated in Ack1 knockdown cells. **B.** Western blotting of  $\beta$ -catenin levels. *Abbreviations:* *Ack1: Activated Cdc42-associated kinase 1*, *TRAIL-R: TNF-related apoptosis-inducing ligand receptor*

#### **4.2.6 Ack1 regulates cell-cell junctions independently of the regulation of TRAIL induced apoptosis**

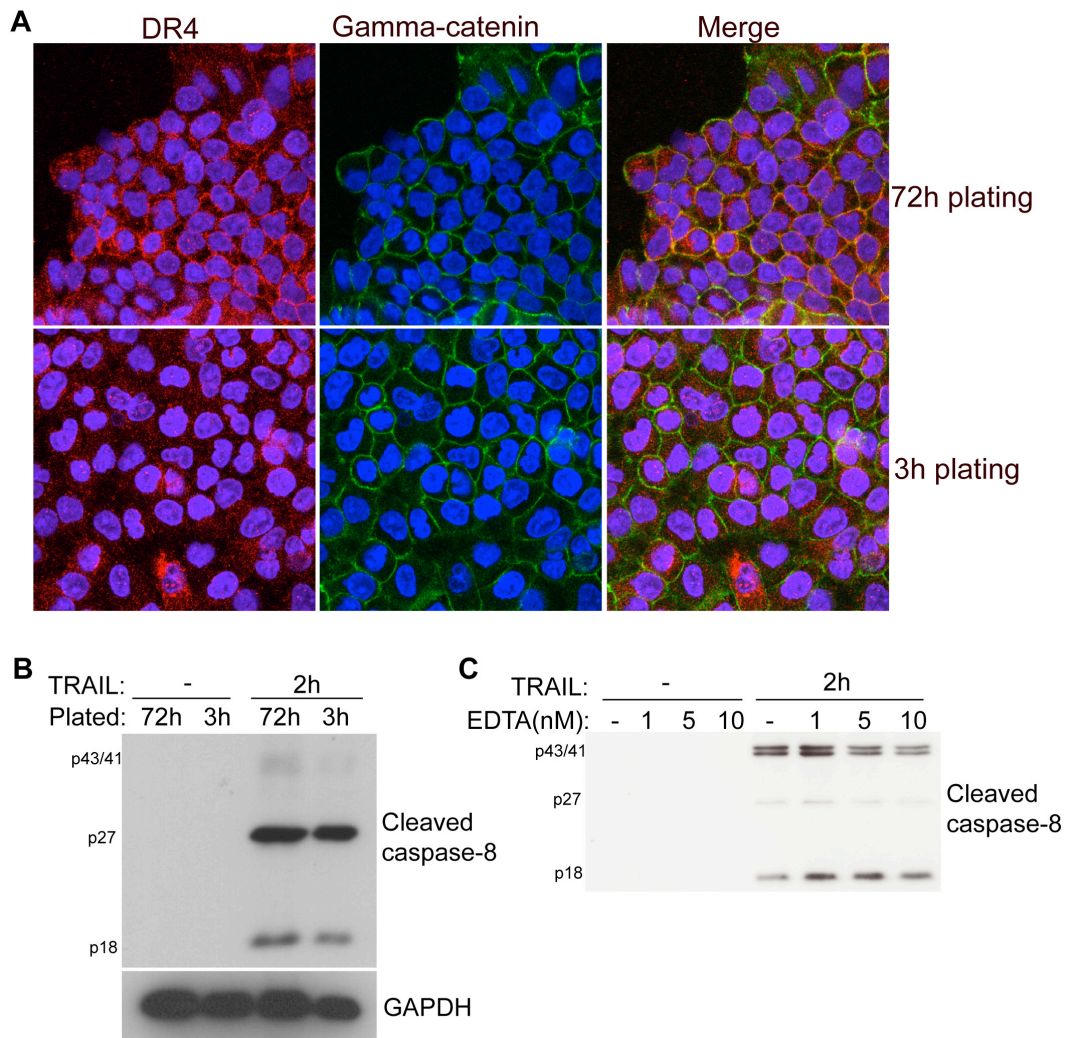
The observation of the co-localisation of TRAIL-R1 and junctional protein as well as the mislocalisation of catenins and Na<sup>+</sup>/K<sup>+</sup> ATPase in Ack1 knockdown cells, raised the question whether cell-cell junction stability and integrity could be important for TRAIL induced apoptosis. In efforts to determine a possible role of cell-cell junctions in TRAIL induced apoptosis, different assays disturbing the cell contacts were used.

Cell-cell junctions are highly dependent on calcium. By depleting the cell culture media of calcium, cell-cell junctions are disrupted. NCI-H460 cells were let to adhere for 48h, to allow the junctions fully form. EDTA was thereafter added to the culture media for 3h, chelating calcium. Following calcium depletion, the cells were treated with TRAIL and the apoptotic response was assessed. Western blot results of cleaved caspase-8 levels show that calcium withdrawal did not affect the apoptotic response (Figure 22C).

Another way of investigating if TRAIL-R localisation on cell-cell contacts are important for TRAIL induced cell death is to compare the apoptotic response between cells adhered for a short time with cells adhered for a longer time. NCI-H460 cells were left to attach to a cell culture plate for 72h so the cells reached an approximate confluency of 80%. The cells were thereafter either left adhered or trypsinised and plated again and allowed to attach for 3h. Following the 72 or 3h attachment time, the cells were treated with TRAIL for 2h. Immunocytochemistry staining show that the  $\gamma$ -catenin-containing cellular junctions are forming after 3h of attachment, however TRAIL-R1 has not translocated back to the junctional compartments (Figure 22A). Western



blotting results show that there is no difference in the cell death response depending on the time adhered (Figure 22B). These two experiments suggest that the cell-cell junctions are not important for the cell death response.



**Figure 22. Cell-cell contact localisation of TRAIL-R1 is not required for TRAIL induced apoptosis.**

**A.** Immunocytochemistry of  $\gamma$ -catenin (green) and TRAIL-R1 (red) in sparse compared to dense plated cells. **B.** Apoptotic response between cells attached for 3h and 72h was compared. The duration of cell attachment did not affect caspase-8 cleavage after TRAIL treatment. **C.** NCI-H460 cells were cultured in cell culture containing EDTA, chelating calcium, for 3h followed by TRAIL treatment for 2h. Calcium depletion did not affect the amounts of cleaved caspase-8 in TRAIL treated cells. *Abbreviations: TRAIL-R: TNF-related apoptosis-inducing ligand receptor*

#### 4.2.7 Discussion

Studies have shown that receptor compartmentalisation and internalisation is important for responsiveness to TRAIL. Both clathrin dependent and independent mechanisms for the internalisation of the TRAIL-receptors have been suggested [74, 86, 154]. However the results published have been conflicting and the trafficking of the TRAIL receptors is still not fully understood. Ack1 is known to interact with the heavy chain of clathrin and the adaptor protein AP-2. A role for Ack1 in clathrin induced apoptosis of the ErbB receptors have previously been suggested by others [120]. We therefore wanted to investigate if Ack1 controls the internalisation of the TRAIL receptors via clathrin mediated endocytosis. The widely used drug Chlorpromazine was used to inhibit clathrin mediated endocytosis followed by TRAIL treatment with TRAIL for 2.5h. Chlorpromazine treatment alone did not induce caspase-8 cleavage. The reduced sensitivity to TRAIL in Ack1 knockdown cells was not restored upon CPZ treatment, suggesting that Ack1 regulates TRAIL induced apoptosis independently of clathrin mediated endocytosis (Figure 18). These results agree with one previous report claiming that the internalisation of the TRAIL receptors is not required for cell death [86].

A correlation between low cell surface expression of the TRAIL receptors and TRAIL resistance has been confirmed in human breast epithelial cells (34). We therefore wanted to investigate if Ack1 knockdown may affect the cell surface expression of the TRAIL receptors. The cell surface expression was investigated by flow cytometry analysis using PE-conjugated antibodies binding to the extracellular domain of TRAIL-R1 and -R2 respectively. In addition, biotin labelling of cell surface protein, followed by immunoprecipitation using streptavidin beads and immunoblotting was used to confirm the flow cytometry data. Results show that there is no difference in the cell surface expression of the TRAIL receptors in Ack1 knockdown cells (Figure 19). The total level of TRAIL-R1 was not equal in the Biotin-IP experiment so it was therefore not possible to confirm the flow cytometry data in this case.

To further investigate the sub-cellular localisation of the TRAIL receptors, ICH experiments were performed. The TRAIL sensitive, lung cancer cell line, NCI-H460 was used for all ICH in this work. The cells are suitable due to the fact that they express relatively high levels of Ack1 and TRAIL-R1, estimated by western blotting (data not shown). ICH staining of TRAIL-R1 showed that the receptor is mainly localised at cell-cell contacts or in perinuclear compartments (Figure 20). The  $\text{Na}^+/\text{K}^+$  ATPase was used as a basolateral membrane marker and has previously been shown to co-localise with cell-cell junctional proteins [155]. Co-staining shows that TRAIL-R1 co-localises with  $\text{Na}^+/\text{K}^+$  ATPase at cell-cell contacts, which has not previously been reported (Figure 20). The sub-cellular localisation of TRAIL-R1 in control-transfected cells was compared to Ack1 knockdown cells, and  $\gamma$ -catenin and  $\text{Na}^+/\text{K}^+$  ATPase were used as markers for the cell-cell junctions. Results show that TRAIL-R1 co-localises with both  $\gamma$ -catenin and  $\text{Na}^+/\text{K}^+$  ATPase in control cells, whereas all three proteins were mislocalised in Ack1 knockdown cells. The  $\gamma$ -catenin positive junctions appear disorganised, ZO-1 positive tight junctions disrupted and TRAIL-R1 no longer localise distinctly at cell-cell contacts (Figure 21A-B). Since the flow cytometry data show that the cell surface expression of TRAIL-R1 is normal in Ack1 knockdown cells, the TRAIL-R1 is most likely localised more diffusely over the plasma membrane, compared to control cells which seem to have TRAIL-R1 concentrated at cell-cell contact areas. By analysing the confocal images in an orthogonal view it was possible to see that  $\gamma$ -catenin and TRAIL-R1 were localised at the apical side of the cells in Ack1 knockdown cells. Control transfected cells show  $\gamma$ -catenin mainly at the basolateral side of the cell (Figure 21A). Mislocalisation of junctional protein is a hallmark of disrupted polarity in epithelial cells (reviewed by [156]), suggesting that Ack1 may play a role in maintaining polarity. A role for Ack1 in regulating cell-cell junctions or polarity has not previously been described and would therefore be very interesting to investigate further. Cdc42 is a known regulator of polarity, for example by directing vesicle trafficking of proteins to distinct domains of the plasma membrane [136-138]. Additionally, Cdc42 has been suggested to

regulate cell-cell junction stability or formation, however the exact mechanism is not understood (Reviewed by [157]. Activation of Cdc42 in endothelial cells promotes reannealing of adherens junctions [158] and stabilises AJs by regulation of apical endocytosis in drosophila [142]. It could therefore be possible that Ack1 regulates cell-cell junctions, and potentially polarity, downstream of Cdc42. The junctions were further investigated by ICH. Staining of the tight junction marker ZO-1 show that the protein was considerably down regulated in Ack1 knockdown cells (Figure 21C) also confirmed by western blotting (Figure 21D). In addition  $\beta$ -catenin was clearly localised at cell-cell contact at the lateral side of the cells in control cells, whereas  $\beta$ -catenin was significantly mislocalised in Ack1 knockdown cells. The junctions appear to be destabilised with a more irregular and spiky appearance.  $\beta$ -catenin is known to be degraded when cell-cell junctions are disrupted, however the total protein level of  $\beta$ -catenin was however not affected by the Ack1 knockdown (Figure 21E) indicating that  $\beta$ -catenin containing junctional complexes are still formed. From the experiments performed it is not clear how Ack1 regulates stability and formation of adherence and tight junctions respectively.

Furthermore, assays where the junctions were disrupted were performed in effort to elucidate whether intact cell-cell junctions are required for TRAIL induced apoptosis. To start with, the cell death response was compared between cells adhered for 72h with cells only plated for 3h. There was no difference in cleaved caspase-8 levels depending on the adhesion time (Figure 22B). ICH staining comparing the cells attached for 72 or 3h, show clear staining of both TRAIL-R1 and  $\gamma$ -catenin at cell-cell contracts in cells adhered for 72h. In contrast, the cells plated for 3h display a much more diffuse staining pattern of TRAIL-R1 although the  $\gamma$ -catenin containing junctions were formed (Figure 22A). In addition, junctions were also disrupted by Calcium depletion, since many of the protein-protein interactions forming the cellular junctions are highly dependent on calcium. EDTA was added to the cell culture media in order to deplete the cells of Calcium, the cells were thereafter treated with TRAIL. No difference in cell-death was detected after calcium depletion



compared to cells cultured with calcium containing media (Figure 22C). These two assays suggest that localisation of TRAIL-R1 on cell-cell junctions and the junctions themselves are not required for TRAIL-induced apoptosis.

### ***4.3 Ack1 is required for DISC formation, receptor clustering and lipid raft translocation of the TRAIL-receptors***

#### **4.3.1 Background and aims**

In order to further investigate the molecular mechanism by which Ack1 regulates TRAIL induced apoptosis a series of biochemical assays, exploring events occurring after TRAIL ligand binding, such as clustering and DISC formation were performed.

#### **The aims were:**

- a) Analyse the formation of the DISC complex in the presence or absence of Ack1.
- b) Study the oligomerisation of the TRAIL receptors following ligand binding.
- c) Elucidate the lipid raft localisation of the TRAIL receptors and further elucidate if Ack1 might regulate the lipid raft localisation of the receptors.

#### **4.3.2 Ack1 knockdown leads to impaired DISC formation**

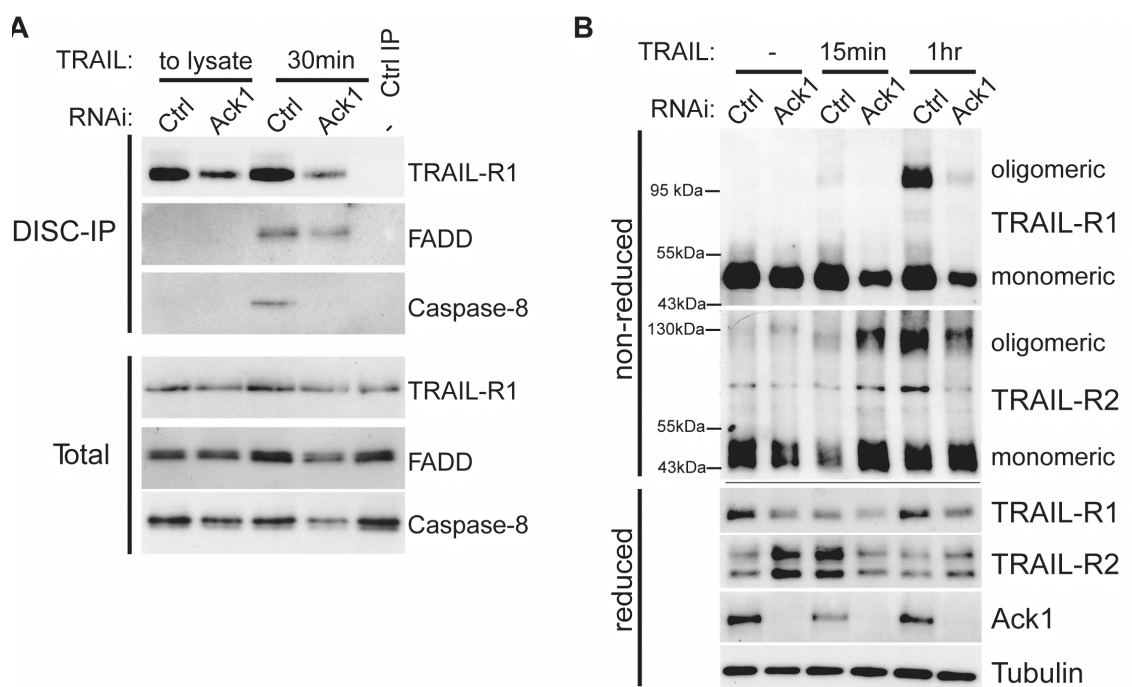
The formation of the DISC is crucial for the initiation of the apoptotic response in a cell following ligand binding to a death receptor. A DISC formation assay was performed to investigate if Ack1 is required for the assembly of the complex. MCF10A cells were treated with biotin-conjugated TRAIL for 30 min at 37°C, allowing TRAIL to bind to the receptors on the cell surface and the subsequent formation of the DISC. The cells were thereafter lysed and the DISC was co-immunoprecipitated (co-IP) with streptavidin beads binding biotin with high affinity. In order to detect the total receptor levels, TRAIL was instead added after the cell lysis in some samples, allowing TRAIL to bind all receptors

present in the cells, without the formation of DISCs (Figure 23A Lane 1-2). Additionally, as a negative control, TRAIL was omitted completely in one sample in order to rule out unspecific binding to the beads alone. Input samples were also collected directly after lysis prior to immunoprecipitation. The co-IP and input samples were subject to SDS-PAGE and western blotting. No protein was detected in the control-IP, confirming that the immunoprecipitation worked satisfactory without unspecific binding of protein to the beads. There was a slight decrease in the total levels of TRAIL-R1 in Ack1 knockdown cells (Figure 23A Lane 1-2), and less TRAIL-R1 were therefore immunoprecipitated. The recruitment of the adaptor protein FADD, which is crucial for the formation of the DISC complex, was not altered in Ack1 knockdown cells. However an impaired recruitment of full-length caspase-8 to the complex was seen in the Ack1 knockdown cells, indicating that Ack1 is required for the recruitment of caspase-8 and complete formation of the DISC (Figure 23A Lane 3-4).

#### **4.3.3 Ack1 is required for oligomerisation of TRAIL-R1**

The oligomerisation and clustering of the TRAIL receptors are important for the DISC formation and subsequent downstream signalling (reviewed in [159]). Since the DISC formation was impaired in the absence of Ack1, we wanted to investigate whether this defect could be due to a defective oligomerisation of the receptors. TRAIL induced oligomerisation of the TRAIL receptors were investigated by western blotting. By omitting the reducing agent DTT from the SDS sample buffer and avoid boiling of the cell lysates, oligomeric forms of the TRAIL receptors can be detected. MCF10A cells, either transfected with control or Ack1 siRNA, were treated with TRAIL for the time points indicated. The cells were harvested, lysed and resuspended in SDS-sample buffer with or without DTT. Samples were run on a SDS-PAGE gel followed by western blotting. The non-reduced samples were blotted for TRAIL-R1 and -R2 (Figure 23B). The monomeric form of TRAIL-R1, around 43kDa, was detected as well as a larger Mw band around 100 kDa constituting oligomeric receptor complexes. Results show that a weak band representing the oligomeric form of TRAIL-R1 can be detected already after 15 min of TRAIL treatment in control transfected cells (Figure 23A, lane 3). Furthermore, after 1h of TRAIL treatment there is a

notably increased amount of oligomerised TRAIL-R1 in control cells. In contrast, significantly lower levels of oligomerised forms of TRAIL-R1 is detected in Ack1 knockdown cells. In addition, total levels of TRAIL-R1 are reduced in the Ack1 knockdown cells following TRAIL treatment, suggesting that the stability of the receptor may be decreased. These results show that Ack1 is required for the oligomerisation of TRAIL-R1. On the other hand, TRAIL-R2 oligomerisation seems to be un-affected by Ack1 knockdown (Figure 23B). Two oligomeric complexes are seen, the bands are however also detected in non-treated samples, suggesting that these form are not solely induced by TRAIL.



**Figure 23. Ack1 knockdown impair the recruitment of caspase-8 to the DISC and the clustering of TRAIL-R1**

**A.** The DISC was co-immunoprecipitated by treating MCF10A cells with biotin-TRAIL for 30 min thereafter lysed and purified with streptavidin beads. In lane 1-2, TRAIL was added to the cleared lysate. No TRAIL was added to the Control IP. The recruitment of FADD was not affected however recruitment of caspase-8 is impaired in Ack1 knockdown cells. **B.** Monomeric and oligomeric forms of the TRAIL receptors were detected after TRAIL treatment by running SDS-PAGE samples without the reducing agent DTT. Reduced samples were run as a control of the total receptor levels. *Abbreviations: DISC: Death inducing signalling complex, DTT:*

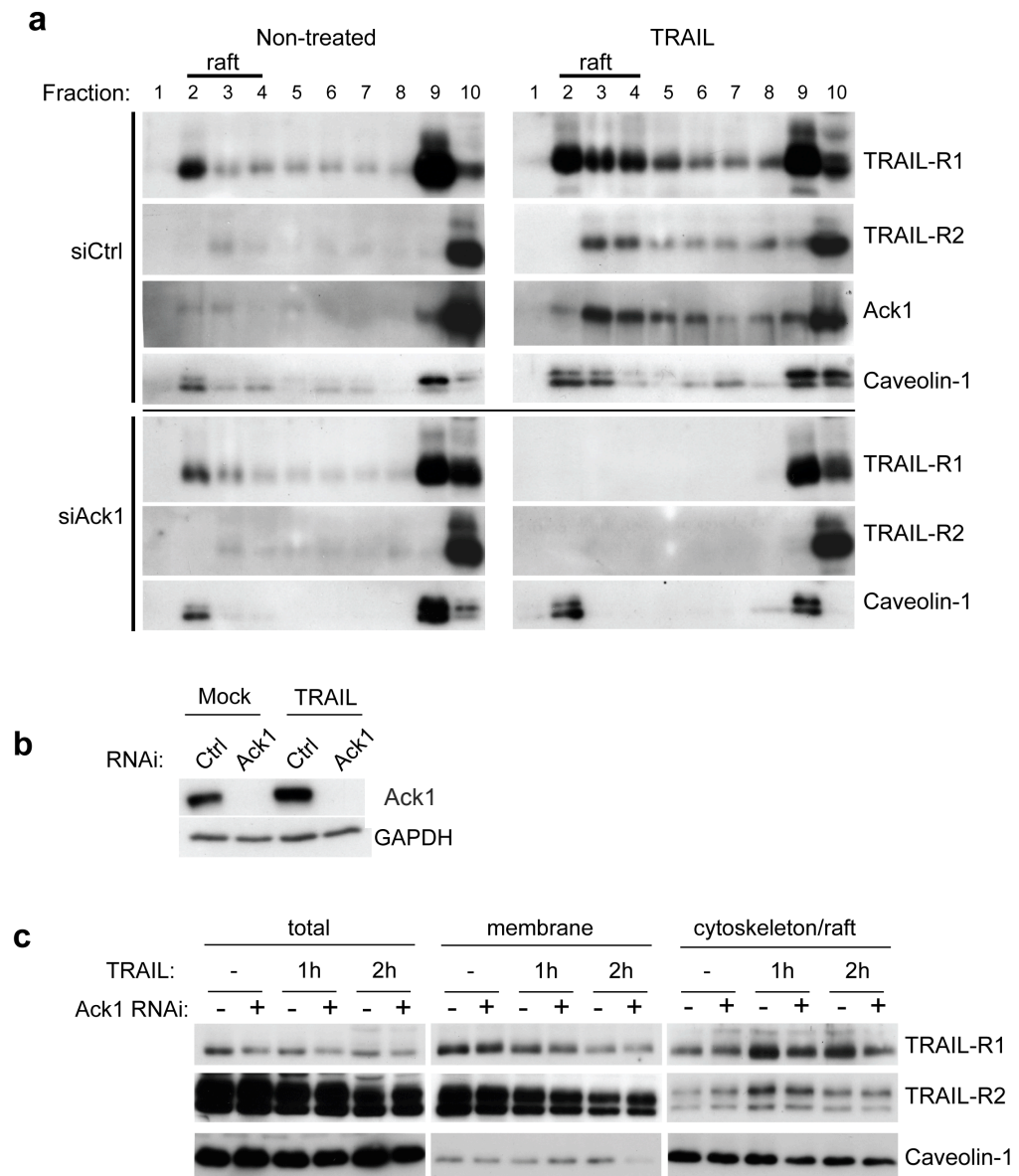
*Ditiotreitol, FADD: Fas-Associated protein with death domain, IP: Immunoprecipitation, TRAIL: TNF-related apoptosis-inducing ligand, TRAIL-R: TRAIL Receptor*

#### **4.3.4 Ack1 knockdown leads to impaired TRAIL induced lipid raft translocation of TRAIL-R1 and TRAIL-R2**

The sub-cellular localisation of the TRAIL-receptors on the plasma membrane has been shown to be of significant importance for TRAIL induced signalling. Dynamic micro-domains in the plasma membrane, enriched with sphingolipids and cholesterol, called lipid rafts, have been suggested to be essential for the regulation of TRAIL receptor signalling and a link between receptor clustering and lipid raft localisation of the receptors have been proposed [99-101]. In order to investigate the membrane localisation of the TRAIL receptors, lipid rafts were isolated using sucrose gradient and ultracentrifugation. Cell lysates were mixed with a 85% sucrose solution and thereafter overlaid with 35% and 5% sucrose followed by ultra-centrifugation at 38 000 rpm for 18 h at 4°C. During ultracentrifugation the lipid rafts “float” to the lighter fractions whereas non-raft compartments stay in the heavier fractions. After ultra-centrifugation, 10 fractions from top to bottom were collected. The fractions were mixed with sample buffer and subjected to western blotting. Caveolin-1 was used as a lipid raft marker and was detected in fraction 2-4 which were considered to be the raft fractions (Figure 24A). Some Caveolin-1 was also detected in the non-raft fractions, possible due to excessive shearing of the raft when using the homogeniser. Results show that in the non-treated cells; both TRAIL-R1 and R2 were present in the lipid raft fractions similarly in the control and knockdown cells. A significant translocation of the receptors towards the lipid raft fraction after 1h TRAIL treatment was seen in the control transfected cells. In contrast, the raft localisation of the TRAIL-Rs was completely lost and the receptors were only detected in the heavier non-raft fractions in the Ack1 knockdown cells following TRAIL treatment. These results suggest that Ack1 is required for TRAIL dependent translocation of TRAIL-R1 and R2 to the lipid rafts. In addition, a TRAIL dependent translocation of Ack1 towards the lipid raft enriched fractions was also observed. A slight up-shift in the molecular weight

of Ack1 in the lighter fractions was also noticed, suggesting that Ack1 might be post-translationally modified in the lipid rafts, possibly by tyrosine phosphorylation, although this remains to be demonstrated (Figure 24A).

To further confirm the translocation of the TRAIL receptors to lipid rafts, MCF10A cells were fractionated into sub-cellular fractions using a kit containing buffers with increasing detergent strength. The cells were treated with TRAIL for 1 or 2h. The total protein, the membrane fraction and the cytoskeleton/lipid raft associated fraction were analysed by western blotting. Caveolin-1 was used as a lipid raft marker and was enriched in the cytoskeleton/raft fraction as compared to the membrane fraction (Figure 25D). In control cells, TRAIL treatment leads to decreased levels of both TRAIL-R1 and R2 in the membrane fraction, whereas an increase is seen in the cytoskeleton/raft fraction. An increase of TRAIL-R2 in the cytoskeleton/raft fraction was only seen after 1h of treatment, suggesting that the lipid raft localisation is transiently trafficked. In Ack1 knockdown cells, the decrease of the TRAIL receptors in the membrane fraction was comparable to control cells. However, the increase of TRAIL-R1 in the cytoskeleton/raft fraction was not as high as compared to control cells (Figure 25B). The TRAIL-R2 levels in the cytoskeleton/lipid raft fraction were similar to the one of control cells. These results further support the model that Ack1 promotes lipid raft localisation, of at least of TRAIL-R1, after TRAIL ligand binding.

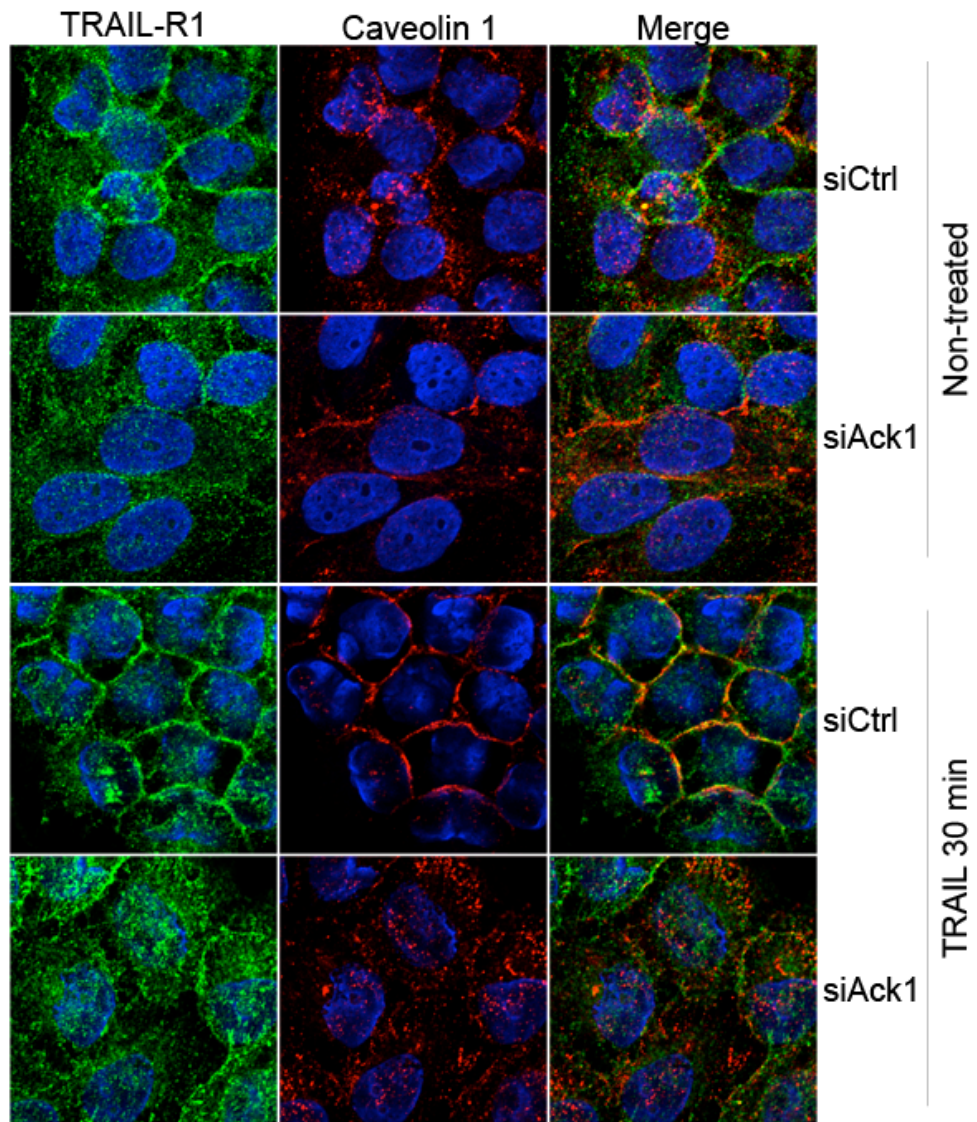


**Figure 24. TRAIL induced translocation of the TRAIL receptors to lipid rafts are dependent of Ack1**

**A.** MCF10A cells were transfected with control or Ack1 siRNA and treated with TRAIL for 1h where indicated. Lipid rafts were isolated using sucrose gradient ultracentrifugation. The detergent resistant lipid raft fractions were enriched in fraction 1-3. Soluble proteins were in the last heavy fractions. Cav1 was used as a raft marker. **B.** Confirmation of Ack1 knockdown efficiency. **D.** MCF10A cells in the absence of presence of Ack1 were treated with TRAIL for 1-2h and thereafter subject to subcellular fractionation using a detergent kit. TRAIL treatment leads to a decrease of both TRAIL-R1 and TRAIL-R2 in the membrane fraction. TRAIL-R1, and TRAIL-R2 to some extent, was translocated to the cytoskeleton/raft associated fraction following TRAIL treatment. Cav1 was used as a lipid raft marker. *Abbreviations: Cav1: Caveolin 1, TRAIL-R: TNF-related apoptosis-inducing ligand receptor*

#### **4.3.5 TRAIL-R1 and Caveolin-1 co-localise following TRAIL treatment**

To further elucidate the role of Ack1 in lipid raft dynamics and translocation of the TRAIL-R1, ICH co-staining of TRAIL-R1 and Caveolin-1 were performed. In non-treated control cells, TRAIL-R1 is mainly localised at cell-cell contacts whereas the staining pattern in Ack1 knockdown cells is more dispersed, as previously shown in (Figure 20). Caveolin-1 is partially located near the cell-cell contacts and in the cytosol in both control and knockdown cells and a partial co-localisation of TRAIL-R1 and Caveolin-1 was seen. Following 30 min TRAIL treatment, both Caveolin-1 and TRAIL-R1 are accumulated at cell contact areas and co-localisation of the two proteins is notably increased in the control cells. In contrast, in Ack1 knockdown cells both proteins show diffuse staining in the whole cell (Figure 25). These results confirm that TRAIL-R1 translocates to Caveolin-1 containing compartments after TRAIL treatment and that Ack1 is required for this event.



**Figure 25. TRAIL-R1 and Caveolin-1 accumulate on the plasma membrane after TRAIL treatment.**

NCI-H460 cells were transfected with non-targeting siRNA or siRNA targeting Ack1 and thereafter left untreated or treated with TRAIL for 30min. ICH staining of Caveolin-1 (Red) and TRAIL-R1 (Green) was performed. Confocal single plane images were acquired at 63x magnification. *Abbreviations: Ack1: Activated Cdc42-associated kinase 1, TRAIL-R: TNF-related apoptosis-inducing ligand receptor, ICH: Immunohistochemistry*



#### 4.3.6 Discussion

In this section, the mechanism by which Ack1 may regulate TRAIL induced apoptosis has been investigated. Results show that Ack1 knockdown leads to an impairment of several early events during TRAIL ligand binding to the receptors.

The DISC formation assay is a useful assay when investigating the most upstream events in death receptor signalling pathways. The recruitment of FADD to the DISC was comparable in control and knockdown cells. However the binding of full-length caspase-8 to FADD is impaired in Ack1 knockdown cells. Since biotinylated TRAIL, binding both TRAIL-R1 and R2, is used in the DISC assay, it is not possible to distinguish from the DISC formation of homo and heteromeric complexes of the receptors. In order to be able to do that, receptor selective antibodies binding the extracellular domain of the receptors need to be used. The reduced recruitment of caspase-8 to the DISC suggests that Ack1 regulates TRAIL induced apoptosis at an early stage of the signalling cascade (Figure 23B). Clustering and oligomerisation of the TRAIL receptors has been shown to be crucial for the efficient formation of the DISC and subsequent downstream apoptotic signalling [98, 101]. The oligomerisation of the receptors was investigated by western blotting of protein lysates without the addition of reducing agent. Oligomeric complexes of TRAIL-R1 were beginning to be formed already after 15min of TRAIL treatment and after 1h significant levels were detected. In contrast, considerably less oligomeric forms were detected in the Ack1 knockdown cell, suggesting that Ack1 is required for the oligomerisation of the receptor, possibly causing the impaired caspase-8 recruitment to the DISC complex (Figure 23A). The oligomerisation of TRAIL-R2 does not seem to be affected by Ack1 knockdown, suggesting that the two receptors might be regulated differently. Little is known about whether TRAIL-R1 and R2 have distinct roles; it is however known that the two receptors are differentially modified post-translationally. TRAIL-R1 can be palmitoylated and the modification has been shown to be crucial for lipid raft localisation, receptor oligomerisation and subsequent apoptosis [107].

The disrupted DISC and impairment of the oligomerisation of TRAIL-R1 in Ack1 silenced cells lead to the question of whether the lipid raft could be involved. A link between oligomerisation/clustering of the receptors and the lipid rafts has been suggested although it is not yet fully understood. Others have reported that clustering and localisation in lipid rafts do correlate, however it is not clear if increased clustering leads to lipid raft localisation, or conversely, that the receptors need to be in the lipid rafts in order to effectively oligomerise and cluster[82, 101]. The discrepancy in clustering between TRAIL-R1 and R2 may suggest that TRAIL-R1 is more dependent on lipid raft localisation in order to fully oligomerise (Figure 23B).

The lipid rafts were investigated by ultra-centrifugation of protein lysates in a sucrose gradient. This is the most widely used biochemical method to study the lipid rafts. The lipid rafts are detergent resistant at low temperatures (4°C) and can thereby be isolated from other soluble membrane compartments. The lipid rafts “float” to the low density fractions formed, whereas the non-raft fractions are found in heavy density fractions. The fractions from the sucrose gradient were analysed by SDS-PAGE and western blotting. In the non-treated cells, the majority of the TRAIL receptors were located in the soluble non-raft fraction, although there were also receptors detected in the lipid rafts to some degree (Figure 24A). The localisation of both TRAIL-R1 and R2 was comparable in control and Ack1 knockdown cells. After 1h of TRAIL treatment, there was a clear translocation of TRAIL-R1 and R2 toward the lipid raft enriched fractions, showing that TRAIL induces the relocation of the receptors to the rafts. These results confirm the reports by others claiming that redistribution of the TRAIL receptors to lipid raft is TRAIL dependent and leads to increased sensitivity to TRAIL [103]. In contrast, knockdown of Ack1 lead to a complete loss of TRAIL-R1 and R2 in the lipid raft fractions after TRAIL treatment (Figure 24). There is also an up-shift of the Ack1 protein in the lighter lipid raft fractions, suggesting that Ack1 localised in lipid raft is post-translationally modified, possibly by phosphorylation. It would have been interesting to also blot for phosphorylated

Ack1; unfortunately the available pAck1 antibody does not work on endogenous protein.

In addition, immunocytochemistry experiments analysing TRAIL-R1 and Caveolin-1 localisation in NCI-H460 cells with or without TRAIL treatment was performed. Confocal imaging shows that after a short TRAIL treatment (30min), both TRAIL-R1 and Caveolin-1 accumulate at or close to the cell surface and a considerably higher percentage of the proteins co-localise. The accumulation of TRAIL-R1 and Caveolin-1 at cell-cell contacts is most likely a transient effect since decreased membrane localisation of the receptors was seen after 1h or longer in the sub-cellular fractionation experiment performed (Figure 24D).

It would have been very interesting to investigate whether Ack1 and TRAIL-R1 interacts. ICH experiments have been performed where the Ack1 and TRAIL-R1 have been co-stained. Unfortunately, the Ack1 antibody is not ideal for ICH staining of endogenous protein so no conclusion could be made from the staining. The Ack1 antibody detects overexpressed protein much more efficiently, however due to poor transfection efficiency the ICH experiments with ectopically expressed Ack1 failed. An alternative way to show that Ack1 and TRAIL-R1 interacts would be to perform an IP. However, technical problems with the IPs using the Ack1 or DR4 antibody as a capturing antibody limited the possibility to perform IP with endogenous protein.

## **Chapter 4 – Final Discussion**

### ***4.4 Summary***

This work shows that Ack1 is required for TRAIL induced apoptosis in human epithelial cells. Lipid raft localisation and oligomerisation of TRAIL-R1, and formation of the DISC, events crucial for the downstream apoptotic signal, are all impaired in Ack1 knockdown cells. siRNA mediated silencing of Cdc42 also results in decreased cell death in response to TRAIL, suggesting that Cdc42 regulates TRAIL induced apoptosis dependently or independently of Ack1. We suggest that Ack1 regulates TRAIL induced apoptosis by promoting the TRAIL-dependent translocation of TRAIL receptors to lipid rafts, which has previously been shown to be very important for the transduction of TRAIL induced death signals. We also note that TRAIL-R1 localises to cell-cell contacts and that the junction integrity is affected by the absence of Ack1. The role of Ack1 in regulating cell-cell junctions appears independent of its role in regulating TRAIL induced apoptosis.

### ***4.5 Final discussion and implications***

TRAIL induced apoptosis has been intensively studied in recent years. TRAIL is considered to be a promising agent for cancer treatment, due to its selective toxicity against tumour cells. However, many cancer cells develop resistance to TRAIL. Several mechanisms for TRAIL resistance have been proposed. The significance of the different mechanisms varies to a large extent between cell types. Understanding the mechanisms by which resistance is acquired will be critical for the therapeutic use of TRAIL in cancer therapy.

In this thesis, a novel regulatory role of Ack1 in apoptosis, death receptor signalling and lipid raft trafficking is presented, contributing further to the understanding of the molecular regulation of TRAIL receptor signalling.

We suggest that Ack1 regulates TRAIL induced apoptosis by promoting the translocation of the TRAIL receptors to lipid rafts after TRAIL treatment. Lipid

raft localisation has been shown to promote apoptosis and sensitise cells to TRAIL [160]. Many studies of the role of lipid rafts in death receptor signalling have been published, however the mechanistic insights explaining how the rafts are regulated and the dynamics of the death receptors in raft versus non-raft compartments is poorly understood. Ack1 is here identified as a key protein in TRAIL receptor dynamics. The precise molecular mechanism, by which Ack1 regulates lipid raft and receptor dynamics have not been fully elucidated in this work. Although, it is evident that Ack1 knockdown leads to disruption of a series of events early in the signalling cascade after TRAIL ligand binding, such as oligomerisation of TRAIL-R1 and the caspase-8 recruitment to the DISC (Figure 23).

Since Ack1 is a tyrosine kinase, it would be of great interest to investigate if kinase activity of Ack1 is required for the regulation of the TRAIL receptors. Tyrosine phosphorylation of Fas, has been shown to be crucial for the oligomerisation of the receptors, amplifying the cell death response [161]. To our knowledge, no kinase has previously been shown to directly phosphorylate the TRAIL receptors. It would be very interesting to investigate if Ack1 phosphorylates the TRAIL-receptors or any other proteins involved in DISC formation and thereby possibly affect the sensitivity to TRAIL. This could be investigated by ectopic expression of a kinase dead Ack1 mutant. A kinase dead mutant, together with other mutants disrupting different interaction sites in Ack1, such as clathrin and Cdc42 binding have been generated. Unfortunately, due to technical difficulties, it has not been possible to express the mutants in an Ack1 knockdown background. Transfecting the cells twice, with siRNA and plasmid DNA, either proved to be toxic for the cells or the ectopic expression was not successful. By using a cell line that is easier and more tolerant to transfection, the experiment might be successful.

Interestingly, knockdown of the Rho GTPase, Cdc42, also resulted in lower levels of cleaved caspase-8 following TRAIL treatment (Figure 17). These results suggest that Cdc42 also plays an important role in TRAIL signalling. Cdc42 is a known effector of Ack1, we therefore hypothesise that the role of

Ack1 in TRAIL induced apoptosis is Cdc42 dependent. One way of proving this model would be to ectopically overexpress an Ack1 mutant with a disrupted Cdc42 binding domain in an Ack1 knockdown background. If the Ack1 mutant failed to restore the cell death response it would suggest that Ack1 require Cdc42 binding and activation in order to regulate the TRAIL receptors.

While investigating the subcellular location of the TRAIL receptors by immunocytochemistry, it was discovered that Ack1 knockdown cells have destabilised cell-cell junctions (Figure 21). Efforts were made in order to elucidate whether intact cell-cell junctions are important for the TRAIL response. Different assays disrupting the junctions were performed and the results suggest that cell-cell junctions are not required for TRAIL induced apoptosis (Figure 22). It would be interesting to further investigate this novel role of Ack1 in cell-cell junction assembly and stability and elucidate the potential biological significance of this phenotype. Cdc42 regulates the stability and endocytosis of junctional proteins. Madin Darby canine kidney (MDCK) cells expressing dominant-active Cdc42V12 show abnormal tight junction morphology, staining of occludin and ZO-1 show that the junctions were irregular in shape and the thickness varied [162]. It would therefore be likely that Ack1 regulates the junctions together with Cdc42. It would also be interesting to address whether other Cdc42 regulated polarity protein such as Par6 and Arp also are involved in TRAIL induced apoptosis and if the membrane compartmentalisation of the TRAIL receptors requires established cell polarity in epithelial cells.

Ack1 has previously been mainly associated with pro-survival signalling and the protein is overexpressed in variety of human primary tumours in which it has been correlated with a poor prognosis [115]. In contrast, we present a pro-apoptotic role of Ack1 specifically via the death receptor signalling pathway but not via the mitochondrial pathway (Figure 13). It is already known that kinases can have both pro-apoptotic and pro-survival roles, for example c-Abl that can both promote apoptosis [163] and mediate growth-factor mitogenic signalling (reviewed in [164]). It is therefore not entirely surprising that Ack1 has a

multi-faced role as well. Efforts are underway to develop inhibitors against Ack1 for use in cancer treatment. A small molecule inhibitor of Ack1 activity, called AIM-100 (4-amino-5,6-biaryl-furo[2,3-d]pyrimidin), has been shown to inhibit prostate cancer cell proliferation. Inhibition of Ack1 also leads to a suppression of the phosphorylation of the androgen receptor (pTyr267-AR) which is known to be critical for the growth of androgen-independent prostate cancer (AIPC). [165, 166]. Our data suggest that inhibition of Ack1 may have unwanted effects on the apoptotic response of cancer cells to exogenous cell death signalling, which is of relevance in the selection of appropriate therapeutic targeting strategies.

With the discovery of a novel protein, Ack1, which crucial for TRAIL induced apoptosis and potentially other death receptor pathways, we hope to present knowledge contributing to the development of effective cancer therapeutics in the future.

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## **Appendix**

### ***Cell culture media composition***

#### **HELA**

DMEM (Invitrogen)

10% fetal bovine serum (FBS) (v/v) (PAA clone, PAA Laboratories Ltd.)

#### **MCF10A**

DMEM/F12 (Invitrogen)

5% horse serum (v/v) (Invitrogen)

20 ng/ml EGF (Miltenyi Biotech)

0.5 mg/ml Hydrocortisone (Sigma)

100 ng/ml Cholera toxin (Sigma)

10 µg/ml Insulin (Sigma)

#### **NCI-H460**

RPMI-1460

10% FBS (v/v)

#### **SW480**

Leibovitz's L15 media (Invitrogen)

10% FBS (v/v)

Cultured in 100% air

### **Mammalian cell freezing media**

70% FBS (v/v)

20% DMEM (v/v)

10% DMSO (v/v) (Sigma)

## ***Homemade solution and Buffer recipes***

### **SDS-PAGE separation gel**

Allow to polymerise for a minimum of 30min.

Percentage	7.5%	10%	12.5%
H <sub>2</sub> O	5.8 ml	4.9 ml	3.9 ml
0.5 M Tris-HCl (pH 8.8)	3.0 ml	3.0 ml	3.0 ml
30 % acrylamide/bis-acrylamide	2.95 ml	3.7 ml	4.75 ml
10 % SDS	120 µl	120 µl	120 µl
TEMED	16 µl	16 µl	16 µl
25 % APS	16 µl	16 µl	16 µl

### **SDS-PAGE stacking gel**

Use 4% stack for ≤ 10% separation gels and 6% stack for >10% separation gels.

Allow to polymerise for a minimum of 30min.

Percentage	4%	6%
H <sub>2</sub> O	3.6 ml	3.6 ml
0.5M Tris-HCl (pH 6.8)	630 µl	630 µl
30 % Acrylamide/bis-acrylamide	660 µl	1 ml
20 % SDS	25 µl	25 µl
TEMED	10 µl	10 µl
25% APS	5 µl	5 µl

### **3X SDS-sample buffer (Laemmli)**

240 mM Tris-HCl pH 6.8

6% SDS

30% v/v Glycerol

0.06 % w/v Bromophenol blue

150mM DTT add just before use:

1.5 ml (1M) DTT

The buffer was used at 1.5x concentration (1:1 in dH<sub>2</sub>O) unless other stated.

### **To make 10ml**

2.4ml Tris-HCl (1M)

3ml SDS (20%)

3ml Glycerol (100%)

0.006g Bromophenol blue

**10 x SDS-PAGE Running buffer**

150mM Tris base

196 mM Glycine

10% w/v SDS

Make up to 1l with dH<sub>2</sub>O**To make 1l**

30.3 g Trizma base

144 g Glycine

10g SDS

**10x Transfer buffer**

250 mM Tris-base

196 mM Glycine

10% Methanol

**To make 1l**

30.26 g Trizma base

147 g Glycine

Make up to 1l with h<sub>2</sub>O

Prepare 1X solution with 10% Methanol prior to use.

**Stripping buffer**

62.5 mM Tris-HCl (pH 6.8)

2% v/v SDS

100 mM 2-beta mercaptoethanol

**To make 250ml**

15.6 ml Tris-HCl (1M)

25 ml SDS (20%)

1.75 ml 2-beta  
mercaptoethanol**10x TBS**

100 mM Tris base pH 7.6

1.5M NaCl

**To make 1l**

12.1g trizma base

87.7g NaCl

pH to 7.6 with HCl (~40ml)

Top up to 1l with dH<sub>2</sub>O**1x TBST**

10% 10x TBS (v/v)

0.01% Tween-20

**To make 1l**

10 ml 10x TBS

1 ml Tween-20

Make up to 1l with h<sub>2</sub>O

**Colloidal Coomassie**

1g/l Coomassie brilliant blue G250

50% H<sub>2</sub>O (v/v)

27.75% H<sub>2</sub>SO<sub>4</sub> (v/v)

Stir with a magnetic bar for 3h

Filter through a fluted filter add,

11% 10N NaOH (v/v)

15.5% Tri-chloric acid (v/v)

To destain use dH<sub>2</sub>O.

**To make 1l**

1 g Coomassie brilliant blue

500 ml H<sub>2</sub>O

27.75 ml H<sub>2</sub>SO<sub>4</sub>

110 ml 10 N NaOH

155 ml Tri-chloric acid (v/v)

Make up to 1l with h<sub>2</sub>O

**Classical Coomassie**

1g/l Coomassie brilliant blue R250

25% (v/v) 2-propanol

10% (v/v) Acetic acid

filtrate through a fluted filter

**To make 1l**

1 g Coomassie brilliant blue

25 ml 2-propanol

10 ml Acetic acid

Make up to 1l with H<sub>2</sub>O

**Coomassie destainer**

10 % (v/v) Acetic acid

40 % (v/v) Methanol

**Phosphatase and protease inhibitor**

Following additions were added to Triton lysis buffers just prior to use unless other stated.

1mM Sodium Vanadate (Na<sub>3</sub>VO<sub>4</sub>) (New England Biolabs)

1mM Sodium Fluoride (NaF, Sigma)

HALT protease and phosphatase inhibitor cocktail (EDTA free) (Thermo Scientific)

(contains Aprotinin, Bestatin, E-64, Leupeptin, Sodium Fluoride, Sodium Orthovanadate, Sodium Phyrophosphate and Beta-glycerophosphate)

**Triton lysis buffer for TRAIL purification**

20mM Tris-HCl

150mM NaCl

10% Glycerol

1% Triton X-100

1mM PMSF

**TRAIL elution buffer**

300 mM NaCl

100 mM Imidazole

Dissolved in PBS

**Triton lysis buffer for TRAIL receptor clustering assay**

20 mM Tris HCl (pH 7.5)

150 mM NaCl

10% Glycerol

0.2% Triton-X 100

1 mM EDTA

**Triton lysis buffer for DISC formation assay**

20 mM Tris HCl (pH 7.5)

150 mM NaCl

10% Glycerol

1% Triton-X 100

1 mM EDTA

**Triton lysis buffer for isolation of lipid rafts**

10 mM TRIS-HCl (pH 7.5)

150 mM NaCl

5 mM EDTA

1 % Triton x-100

**Triton lysis buffer for Biotin-IP**

20mM Tris-HCl

150mM NaCl

10% Glycerol

1% Triton X-100

**10x Annexin V binding buffer**

0.1 M Hepes pH 7.4

1.5 M NaCl

50 mM KCl

10 mM MgCl<sub>2</sub>

18 mM CaCl<sub>2</sub>

Dissolve in dH<sub>2</sub>O

Prepare 1x solution with H<sub>2</sub>O prior to use

**DNA extraction buffer**

0,2 M Na<sub>2</sub>HPO<sub>4</sub>

0,004% Triton X-100 (v/v)

pH 7.8

**DNA staining solution (PI)**

50 µg/ml Propidium Iodide

0.2 mg/ml RNase A (dissolved in 10 mM Sodium acetate (pH 7.4) in PBS)

**Collagen coating of coverslips**

0.1 % Collagen type I (rat tail, BD Biosciences) (v/v)

Diluted in 0.02M acetic acid.

**Biotinylation buffer for biotin-IP**

50 µl/ml Biotin-7-NHS

50 mM sodium borate

150 mM NaCl

**0.5 M EDTA (pH 8.0)**

84.05 g/l EDTA

Dissolve in dH<sub>2</sub>O

Add NaOH pellets until the EDTA fully dissolve and reaches pH 8.0

**50x TAE Buffer**

2 M Tris-acetate

Glacial acetic acid

acid

0.05 M EDTA pH 8.0

**To make 1l**

242g Trizma base

57.1 ml glacial acetic

100ml EDTA pH 8.0

Top up to 1l with dH<sub>2</sub>O

**10x DNA loading buffer**

50% Glycerol (v/v)

0.2% Orange G (Sigma #0165)

Dissolved in 1xTAE

Top up to 200ml with dH<sub>2</sub>O

**To make 200ml**

100ml glycerol

0.4 g Orange G

4ml TAE (50x)

**100 mg/ml Ampicillin (1000x)**

To make 20ml

2 g Ampicillin

dH<sub>2</sub>O up to 20 ml

Sterile filter and store at -20°C

**50 mg/ml Kanamycin (1000x)**

To make 20 ml

1 g Kanamycin

dH<sub>2</sub>O up to 20 ml

Sterile filter and store at -20°C



**25 mg/ml Chloramphenicol (500x)**

To make 20 ml

0.5 g Chloramphenicol (company?)

Ethanol up to 20 ml

Store at -20°C

**100 mg/ml Spectinomycin (100x)**

To make 20 ml

2 g Spectinomycin

dH<sub>2</sub>O up to 20 ml

Sterile filter and store at -20°C

**LB media**

20g/l LB Broth

(10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl)

Dissolved in dH<sub>2</sub>O

Sterilise by autoclaving

**To make 1l**

20g LB Broth

dH<sub>2</sub>O up to 1l

**LB Agar plates**

35g/l LB agar (Sigma)

Dissolved in dH<sub>2</sub>O

Sterilised by autoclaving.

Cool down to 55°C, add appropriate selective antibiotics

Pour into 10 cm Petri dishes, approximately 20 ml/dish and let to dry for 30 min thereafter stored at 4°C.